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1. That I am acquainted with the Japanese and English language; and

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AND I MAKE SOLEMN DECLARATION conscientiously believing the same to be true and correct.

This 24th day of July, 2002

K. Takizawa

Keita Takizawa

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【発明の名称】　ハイブリッド酵素およびその用途

【特許請求の範囲】

【請求項１】配列番号１に示したアミノ酸配列の一部を含むペプチドにより一部が置換されるか、又は当該ペプチドが挿入されたアミノ酸配列を有するハイブリッド酵素であって、当該ハイブリッド酵素が、前記ペプチドが置換又は挿入される前と同様の酵素活性を有し且つ置換又は挿入されたペプチド部分に当該ペプチドに対して結合能を有する物質が結合すると当該酵素活性が変化する性質を有するハイブリッド酵素。

【請求項２】ペプチドが、配列番号１に示したアミノ酸配列のうちから選ばれた少なくとも６個以上の連続したアミノ酸配列を含むものである請求項１記載のハイブリッド酵素。

【請求項３】ペプチドが、C-反応性蛋白質と結合能を有する物質と結合可能な性質を有するものである請求項２記載のハイブリッド酵素。

【請求項４】ペプチドのアミノ酸配列が配列番号２～５の何れかの配列から選ばれた少なくとも６個以上のアミノ酸配列を含むものである、請求項１記載のハイブリッド酵素。

【請求項５】酵素が、グルコース-6-リン酸脱水素酵素、β－ガラクトシダーゼ又はアルカリホスファターゼである、請求項１記載のハイブリッド酵素。

【請求項６】ペプチドと結合能を有する物質が抗体である請求項１記載のハイブリッド酵素。

【請求項７】請求項１～６の何れかに記載された酵素を含んでなる、C-反応性蛋白質測定用試薬。

【請求項８】更に抗C-反応性蛋白質抗体を含んでなる、請求項７記載の試薬。

【請求項９】請求項１～６の何れかに記載された酵素を含む試薬を含んでなる、C-反応性蛋白質測定用キット。

【請求項１０】更に抗C-反応性蛋白質抗体を含んでなる、請求項９記載のキット。

【請求項１１】請求項１～６の何れかに記載された酵素を用いることを特徴とす

る、C-反応性蛋白質の測定方法。

【請求項１２】更に抗C-反応性蛋白質抗体を組み合わせて用いる、請求項１１記載の測定方法。

【請求項１３】C-反応性蛋白質を含む試料と、請求項１～６の何れかに記載された酵素と、抗C-反応性蛋白質抗体とを接触させた後、当該酵素の活性を測定し、得られた酵素活性値に基づいて試料中のC-反応性蛋白質量を求めることを特徴とする、C-反応性蛋白質の測定方法。

【請求項１４】特定位置にペプチドが挿入または置換されたグルコース-6-リン酸脱水素酵素。

【請求項１５】特定位置が、６個以上のアミノ酸残基を有するペプチドの挿入または置換によっても、グルコース-6-リン酸脱水素酵素活性を保持できる位置である、請求項１４記載の酵素。

【請求項１６】特定位置が、挿入または置換されたペプチドに該ペプチドと結合能を有する物質が結合すると、グルコース-6-リン酸脱水素酵素活性が変化する位置である、請求項１４記載の酵素。

【請求項１７】特定位置が配列番号６で示されるグルコース-6-リン酸脱水素酵素のアミノ酸配列のAsp２９４、Leu３０２－Asp３１０位、Glu３６２位、Ｎ末端及びＣ末端から選ばれる任意の位置である、請求項１４記載の酵素。

【請求項１８】ペプチドがC-反応性蛋白質のアミノ酸配列から選ばれたものである、請求項１４に記載の酵素。

【請求項１９】ペプチドが該ペプチドに特異的に結合する物質を有するものである、請求項１４に記載の酵素。

【請求項２０】請求項１４～１９に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測定用試薬。

【請求項２１】請求項１４～１９に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測定用キット。

【請求項２２】請求項１４～１９に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドを含有する物質の測定方法。

【請求項２３】請求項１４～１９に記載された酵素と、該酵素に挿入または置換

されたペプチドと結合能を有する物質とを組み合わせて用いる、当該ペプチドを含有する物質の測定方法。

【請求項２４】請求項１４～１９に記載された酵素と、該酵素に挿入または置換されたペプチドを含有する物質を含む試料と、当該ペプチドに結合能を有する物質とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドを含有する物質の量を求めることを特徴とする、当該ペプチドを含有する物質の測定方法。

【請求項２５】請求項１４～１９に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用試薬。

【請求項２６】請求項１４～１９に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用キット。

【請求項２７】請求項１４～１９に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定方法。

【請求項２８】請求項１４～１９に記載された酵素と、該酵素に挿入または置換されたペプチドと結合能を有する物質を含む試料とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドと結合能を有する物質の量を求めることを特徴とする、当該ペプチドと結合能を有する物質の測定方法。

【請求項２９】配列番号６で示されるグルコース-6-リン酸脱水素酵素のアミノ酸配列のAsp２９４、Leu３０２－Asp３１０位、Glu３６２位、Ｎ末端及びＣ末端から選ばれる任意の位置に、外来ペプチドが置換又は挿入されたアミノ酸配列を有するハイブリッド酵素をコードする遺伝子。

【請求項３０】請求項２９記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ。

【請求項３１】請求項３０記載の組み換えＤＮＡを含む形質転換体又は形質導入体。

【請求項３２】請求項３１記載の形質転換体又は形質導入体を培養し、グルコース-6-リン酸脱水素酵素の酵素活性を有し、且つグルコース-6-リン酸脱水素酵素に置換又は挿入されたアミノ酸配列に、当該アミノ酸配列に結合能を有する物質

が結合すると、グルコース-6-リン酸脱水素酵素活性が変化する性質を有する蛋白質を回収することを特徴とする、上記性質を有する蛋白質の製造方法。

【請求項３３】配列番号６で示されるグルコース-6-リン酸脱水素酵素のアミノ酸配列のAsp２９４、Leu３０２－Asp３１０位、Glu３６２位、Ｎ末端及びＣ末端から選ばれる任意の位置に、制限酵素により開環され得るアミノ酸配列が挿入あるいは置換されたアミノ酸配列を有する、ハイブリッド酵素をコードする遺伝子。

【請求項３４】請求項３３記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ。

【請求項３５】特定位置に、配列番号１に示したアミノ酸配列から選ばれたペプチドが挿入または置換されたβ－ガラクトシダーゼ。

【請求項３６】特定位置が配列番号３０で示されるβ－ガラクトシダーゼのアミノ酸配列のIle280／Asp281、Val796／Ser797の何れかから選択された部位である請求項３５記載のβ－ガラクトシダーゼ。

【請求項３７】請求項３６記載のハイブリッド酵素をコードする遺伝子。

【請求項３８】請求項３７記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ。

【請求項３９】請求項３８記載の組み換えＤＮＡを含む形質転換体または形質導入体。

【請求項４０】請求項３９記載の形質転換体または形質導入体を培養し、β－ガラクトシダーゼの酵素活性を表し、且つβ－ガラクトシダーゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、β－ガラクトシダーゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法。

【請求項４１】特定位置に、配列番号１に示したアミノ酸配列から選ばれたペプチドが挿入または置換されたアルカリフォスファターゼ。

【請求項４２】特定位置が配列番号３１で示されるアルカリホスファターゼのアミノ酸配列のLys167／168Cys、Cys168／169Tyr、Glu407／408Asp、Lys91／93Thr、Tyr169／177Lysの何れかから選択された部位である請求項４１記載のアルカリ

フォスファターゼ。

【請求項４３】請求項４２記載のハイブリッド酵素をコードする遺伝子。

【請求項４４】請求項４３記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ。

【請求項４５】請求項４４記載の組み換えＤＮＡを含む形質転換体または形質導入体。

【請求項４６】請求項４５記載の形質転換体または形質導入体を培養し、アルカリフォスファターゼの酵素活性を表し、且つアルカリフォスファターゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、アルカリフォスファターゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】

本発明はハイブリッド酵素およびそれを用いた生体試料成分分析測定系に関し、更に詳しくはC-反応性蛋白質（以下ＣＲＰと略記する）のアミノ酸配列の一部分を含むハイブリッド酵素および該ハイブリッド酵素を用いたＣＲＰ測定系、特定位置に外来ペプチドを含有するハイブリッド型グルコース-6-リン酸脱水素酵素(G6PDH)及び該ハイブリッド酵素を用いた各種外来ペプチド測定系に関するものである。

【０００２】

【従来の技術】

抗原抗体反応を利用した生体試料成分分析測定系としては、免疫比濁法、ラテックス凝集法等のホモジニアス測定系と、HPLC法、電気泳動法、放射線免疫測定法、酵素免疫測定法（以下、ＥＩＡ法）等のヘテロジニアス測定系との２つに大別される。いずれの方法においても現在ではほとんど自動化された機器を用いて測定されているが、ヘテロジニアス測定系では固相を用いることから高分子物質測定であれ、低分子物質測定であれ特殊な機器が必須であり、汎用の自動分析機には適用できない。また、ホモジニアス測定系においても、免疫比濁法、ラテッ

クス凝集法以外は特殊な機器が用いられ汎用の自動分析機には適用できない。しかし、現在汎用の自動分析機に応用できるホモジニアスな免疫学的測定系として、酵素を用いたＥＭＩＴ法（IMMUNOASSAYS R. M. Nakamura et. al Alan R. Liss, Inc., New York）が確立され、実用化されている。本測定系は、測定対象物質で化学修飾した酵素を用い、これに該測定対象物に対する抗体が結合すると酵素活性が阻害され低下する事を利用して、測定対象物の量を測定する方法であるが、測定対象物がハプテンなどのように低分子のものに限られるという問題点を有していた。そこで最近では、酵素を使用して高分子物質の測定を可能にするホモジニアスな免疫学的測定系として、遺伝子組み換えにより酵素中に外来ペプチドを挿入してハイブリッド酵素を生成しこれを用いたホモジニアス測定系が考案されている。即ち、当該ハイブリッド酵素中の外来ペプチド部分に抗体等が結合する際に酵素活性が変化することを利用して、そのペプチドあるいはそのペプチドをアミノ酸配列中に含む物質又はそのペプチドと結合能を有する物質の量を測定する方法であり、この方法で用いられる酵素としてはアルカリホスファターゼ（特表平8-507686号）やβ－ガラクトシダーゼ（FEBS Letters434(1998)23-27、FEBS Letters438(1998)267-271）等が知られている。しかしながらこれらの方法に於いては、外来ペプチドが挿入される位置により、当該ハイブリッド酵素が酵素活性を有さない場合、あるいは酵素活性があったとしても結合能を有する物質が該ハイブリッド酵素に結合したときに酵素活性が変化しない場合があること等も知られている。

一方、ＣＲＰは、肺炎球菌の莢膜のＣ多糖体と反応する血清中のβグロブリンであるが、急性期反応蛋白であり組織破壊を伴うような炎症性疾患時等に増加することが知られており、ＣＲＰの測定は、組織中の炎症の早期発見、病気の診断等に有用である。このＣＲＰの測定は、従来、免疫比濁法、ラテックス凝集法、酵素免疫法、放射性免疫法等により行われている。これらのうち、免疫比濁法、ラテックス凝集法は、汎用自動分析装置に適応可能な方法であるが、どちらも濁度変化を検出するものであるため、完全なホモジニアス測定系とは言えず、測定精度の面において問題がある。従って、測定精度が良い完全なホモジニアス系による汎用自動分析装置に適応可能なＣＲＰの微量測定法の開発が望まれている。

【０００３】

【発明が解決すべき課題】

上記した如き状況に鑑み、本発明が解決しようとする課題の１つは、ホモジニアスな比色法で試料中の微量なＣＲＰ測定を可能とする方法を提供することにある。もう一つの本発明が解決しようとする課題は、高分子物質をホモジニアスな系で測定するための方法を提供することにある。

【０００４】

【課題を解決するための手段】

本発明者らは上記課題を解決すべく研究を重ねた結果、上述したハイブリッド酵素を用いれば、高分子の測定対象物を、ホモジニアスな系で測定することが可能であり、また、その検出は比色法で行うことが可能であることから、この方法をＣＲＰの測定に応用すれば、汎用自動分析装置に適応可能なＣＲＰの新たな微量分析法が開発し得ると考え、更に研究を重ねて、ある種の酵素のある位置にＣＲＰ等の被分析物質の一部を含有するハイブリッド酵素を用いることにより上記の課題が解決されることを見出し、本発明に到達したものである。

即ち、本発明は、特定の酵素中にＣＲＰ由来のペプチドを挿入したハイブリッド酵素を用いて、ホモジニアスな比色法で試料中の微量なＣＲＰ測定を可能とする方法を提供する為のハイブリッド酵素として、（１）配列番号１に示したアミノ酸配列の一部を含むペプチドにより一部が置換されるか、又は当該ペプチドが挿入されたアミノ酸配列を有するハイブリッド酵素であって、当該ハイブリッド酵素が、前記ペプチドが置換又は挿入される前と同様の酵素活性を有し且つ置換又は挿入されたペプチド部分に当該ペプチド対して結合能を有する物質が結合すると当該酵素活性が変化する性質を有するハイブリッド酵素、（２）ペプチドが、配列番号１に示したアミノ酸配列のうちから選ばれた少なくとも６個以上の連続したアミノ酸配列を含むものである（１）記載のハイブリッド酵素、（３）ペプチドが、ＣＲＰと結合能を有する物質と結合可能な性質を有するものである（２）記載のハイブリッド酵素、（４）ペプチドのアミノ酸配列が配列番号２～５の何れかの配列から選ばれた少なくとも６個以上のアミノ酸配列を含むものである（１）記載のハイブリッド酵素、（５）酵素が、グルコース-6-リン酸脱水素

酵素(以下G6PDHと略記する場合がある)、β－ガラクトシダーゼ又はアルカリホスファターゼである（１）記載のハイブリッド酵素、（６）ペプチドと結合能を有する物質が抗体である（１）記載のハイブリッド酵素が、有効であることを見出したものである。そして上記のハイブリッド酵素を用いたホモジニアスな比色法で試料中の微量なＣＲＰ測定方法として、（７）（１）～（６）の何れかに記載された酵素を含んでなるCRP測定用試薬、（８）更に抗CRP抗体を含んでなる（７）記載の試薬、（９）（１）～（６）の何れかに記載された酵素を含む試薬を含んでなるCRP測定用キット、（１０）更に抗CRP抗体を含んでなる、請求項９記載のキット、（１１）（１）～（６）の何れかに記載された酵素を用いることを特徴とするCRPの測定方法、（１２）更に抗CRP抗体を組み合わせて用いる（１１）記載の測定方法、（１３）CRPを含む試料と、（１）～（６）の何れかに記載された酵素と、抗CRP抗体とを接触させた後、当該酵素の活性を測定し、得られた酵素活性値に基づいて試料中のCRP量を求めることを特徴とする、CRPの測定方法、を提供するものである。

また本発明では高分子物質をホモジニアスな系で測定するために、外来ペプチドが挿入されても同様の酵素活性を有し、且つ外来ペプチド部分に結合能を有する物質が結合した場合、当該酵素活性が変化し得る、G6PDH含有ハイブリッド酵素として、（１４）特定位置にペプチドが挿入または置換されたG6PDH、（１５）特定位置が、６個以上のアミノ酸残基を有するペプチドの挿入または置換によっても、G6PDH活性を保持できる位置である（１４）記載の酵素、（１６）特定位置が、挿入または置換されたペプチドに該ペプチドと結合能を有する物質が結合すると、G6PDH活性が変化する位置である（１４）記載の酵素、（１７）特定位置が配列番号６で示されるG6PDHのアミノ酸配列のAsp２９４、Leu３０２－Asp３１０位、Glu３６２位、Ｎ末端及びＣ末端から選ばれる任意の位置である（１４）記載の酵素、（１８）ペプチドがCRPのアミノ酸配列から選ばれたものである（１４）記載の酵素、（１９）ペプチドが該ペプチドに特異的に結合する物質を有するのである（１４）記載の酵素、が有効であることを見出したものである。更に（２０）（１４）～（１９）に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測定用試薬、（２１）（１４）～

（１９）に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドを含有する物質の測定用キット、（２２）（１４）～（１９）に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドを含有する物質の測定方法、（２３）（１４）～（１９）記載された酵素と、該酵素に挿入または置換されたペプチドと結合能を有する物質とを組み合わせて用いる、当該ペプチドを含有する物質の測定方法、（２４）（１４）～（１９）に記載された酵素と、該酵素に挿入または置換されたペプチドを含有する物質を含む試料と、当該ペプチドに結合能を有する物質とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドを含有する物質の量を求めることを特徴とする、当該ペプチドを含有する物質の測定方法、（２５）（１４）～（１９）に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用試薬（２６）（１４）～（１９）に記載された酵素を含んでなる、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定用キット、（２７）（１４）～（１９）に記載された酵素を用いることを特徴とする、該酵素に挿入または置換されたペプチドと結合能を有する物質の測定方法、（２８）（１４）～（１９）に記載された酵素と、該酵素に挿入または置換されたペプチドと結合能を有する物質を含む試料とを接触させた後、該酵素の活性を測定し、得られた酵素活性値に基づいて試料中の当該ペプチドと結合能を有する物質の量を求めることを特徴とする、当該ペプチドと結合能を有する物質の測定方法を提供するものである。

また本発明ではある物質或いはこれに結合能を有する物質を検出するための試薬を製造するために、（２９）配列番号６で示されるG6PDHのアミノ酸配列のAsp２９４、Leu３０２－Asp３１０位、Glu３６２位、Ｎ末端及びＣ末端から選ばれる任意の位置に、外来ペプチドが置換又は挿入されたアミノ酸配列を有するハイブリッド酵素をコードする遺伝子が有用であることを見出したものである。更に本発明は、（３０）（２９）記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ、（３１）（３０）記載の組み換えＤＮＡを含む形質転換体又は形質導入体、（３２）（３１）記載の形質転換体又は形質導入体を培養し、G6PDHの酵素活性を有し、且つG6PDHに置換又は挿入

されたアミノ酸配列に、当該アミノ酸配列に結合能を有する物質が結合すると、G6PDH活性が変化する性質を有する蛋白質を回収することを特徴とする、上記性質を有する蛋白質の製造方法、（３３）配列番号６で示されるG6PDHのアミノ酸配列のAsp２９４、Leu３０２－Asp３１０位、Glu３６２位、Ｎ末端及びＣ末端から選ばれる任意の位置に、制限酵素により開環され得るアミノ酸配列が挿入あるいは置換されたアミノ酸配列を有する、ハイブリッド酵素をコードする遺伝子、（３４）（３３）記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ、を提供するものである。

さらには、（３５）特定位置に、配列番号１に示したアミノ酸配列から選ばれたペプチドが挿入または置換されたβ－ガラクトシダーゼ、（３６）特定位置が配列番号３０で示されるβ－ガラクトシダーゼのアミノ酸配列のIle280／Asp281、Val796／Ser797の何れかから選択された部位である（３５）記載のβ－ガラクトシダーゼ、（３７）（３６）記載のハイブリッド酵素をコードする遺伝子、（３８）（３７）記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ、（３９）（３８）記載の組み換えＤＮＡを含む形質転換体または形質導入体、（４０）（３９）記載の形質転換体または形質導入体を培養し、β－ガラクトシダーゼの酵素活性を表し、且つβ－ガラクトシダーゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、β－ガラクトシダーゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法を提供するものである。

さらにまた、（４１）特定位置に、配列番号１に示したアミノ酸配列から選ばれたペプチドが挿入または置換されたアルカリフォスファターゼ、（４２）特定位置が配列番号３１で示されるアルカリホスファターゼのアミノ酸配列のLys167／168Cys、Cys168／169Tyr、Glu407／408Asp、Lys91／93Thr、Tyr169／177Lysの何れかから選択された部位である（４１）記載のアルカリフォスファターゼ、（４３）（４２）記載のハイブリッド酵素をコードする遺伝子、（４４）（４３）記載のハイブリッド酵素遺伝子をベクターＤＮＡに挿入したことを特徴とする新規な組み換えＤＮＡ、（４５）（４４）記載の組み換えＤＮＡを含む形質転換体

または形質導入体、（４６）（４５）記載の形質転換体または形質導入体を培養し、アルカリフォスファターゼの酵素活性を表し、且つアルカリフォスファターゼに置換または挿入されたアミノ酸配列に当該アミノ酸配列と結合能を有する物質が結合すると、アルカリフォスファターゼ活性が変化する性質を有する、タンパク質を回収することを特徴とする、上記性質を有するタンパク質の製造方法を提供するものである。

【０００５】

【発明の実施形態】

本発明のハイブリッド酵素に用いるオリジン酵素としては、一般的に用いられている酵素であればどのようなものを用いてもよいが、アデノシンデアミナーゼ；アルカリ性ホスファターゼ；α－アミラーゼ；細菌ルシフェラーゼ；β－ガラクトシダーゼ；β－ガラクトシダーゼフラグメント；β－ラクタマーゼ；カルボニックアンヒドラーゼ；カタラーゼ；ホタルルシフェラーゼ；グルコースオキシダーゼ；グルコース－６－リン酸脱水素酵素；グルコシダーゼ；ヘキソキナーゼ；西洋ワサビペルオキシダーゼ；インベルターゼ；イソクエン酸デヒドロゲナーゼ；リゾチーム；リンゴ酸デヒドロゲナーゼ；マイクロペルオキシダーゼ；６－ホスホフラクターゼ；及びキサンチンオキシターゼなどが挙げられる。

さらに、アミノ酸配列や遺伝子配列が明らかであるか、明らかにし得るものであり、外来ペプチドにより一部又は複数部が置換されるか或いは外来ペプチドを一部又は複数部に挿入し、当該ペプチドに対して結合能を有する物質が結合すると当該酵素活性が変化するものはすべて使用可能である。中でも、酵素活性が高いもの、安定性がよいもの、比色法で測定できるもの等が好ましく、例えば、Ｇ６ＰＤＨ、β-ガラクトシダーゼ、アルカリホスファターゼ等が挙げられる。また、これらの酵素の遺伝子は、通常一般的に行われるゲノムからのクローニング方法等で入手可能であり、もちろん既にクローニングされた遺伝子や合成ＤＮＡ等を使用することもできる。また、これらの酵素の由来はいかなるものであってもよく、そのアミノ酸配列については本来の酵素活性を有するものであれば１もしくは複数のアミノ酸が欠失、置換あるいは付加された配列であってもよい。

本発明のハイブリッド酵素を作製するために用いられる外来ペプチドとしては

、そのペプチドに結合能を有する抗体やレセプター等の物質が存在するものであればどのようなものを用いてもよく、一般に免疫学的測定系で測定されている、例えば、生体成分であるCRP、IgG、IgA、IgM、Ｃ３、Ｃ４、β２ミクログロブリン等、あるいは、α－フェトプロテイン、CA19-9、前立腺特異抗原(PSA)、癌胎児性抗原(CEA)等の各種ガンマーカー、あるいは、インシュリン、ヒト絨毛性ゴナドトロピン(hCG)、アルブミン、ストレプトリジンＯ（ＳＬＯ）、プロラクチン、副甲状腺ホルモン、甲状腺刺激ホルモン(TSH)等の各種ホルモン、あるいは、Ｂ型肝炎ウイルス(HBV)、Ｃ型肝炎ウイルス(HCV)、ヒト免疫不全ウイルス(HIV)、成人Ｔ細胞白血病ウイルス(HPV)等の各種ウイルス等がその供与体として挙げられる。

本発明の組み換えＤＮＡに用いるベクターとしては、プラスミドベクター及びファージベクター等の原核細胞および／または真核細胞の各種宿主内で複製保持または自己増殖できるものであれば特に限定されないが、例えば当業界において入手可能な、pBR322、pBR325、pUC12、pUC13、pBluescript等の大腸菌由来プラスミド、pSH19、pSH15等の酵母由来プラスミド、pUB110、pTP5、pC194等の枯草菌由来プラスミド等が挙げられる。また、ファージベクターとしてはλファージなどのバクテリオファージ、更には、レトロウイルス、ワクシニアウイルス、核多角体ウイルスなどの動物や昆虫のウイルス等が挙げられる。

本発明のハイブリッド酵素の生産に用いる宿主細胞としては、細菌（例えば大腸菌など）、酵母（例えばサッカロマイセス属など）、動物細胞（例えばチャイニーズハムスター細胞ＣＨＯなど）、昆虫細胞（例えばＢｍＮ４など）が挙げられる。

本発明のハイブリッド酵素を生産するための、組み換えＤＮＡの構築や発現及び精製は、自体公知の方法、例えばMolecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載されている方法に従い行えばよい。

また、発現ベクターとしては、原核細胞および／または真核細胞の各種宿主内で複製保持または自己増殖できるものであって、原核細胞および／または真核細胞の各種宿主細胞中でハイブリッド酵素をコードする遺伝子を発現させる機能、

即ち、目的のハイブリッド酵素を生産させる機能を有するものであれば特に限定されないが、例えば当業界において入手可能ものとして、宿主細胞が大腸菌の場合は、pBR322、pUC12、pUC13、pTrcHis、pTrc99A、pMAL-c2、もしくはその人工的修飾物（該ベクターを適当な制限酵素で処理して得られるＤＮＡフラグメント）等が、宿主細胞が酵母の場合は、プラスミドpRS403、pRS404、pRS413、pRS414、pYES2等が、宿主細胞が動物細胞の場合は、プラスミドpRSVneo ATCC37224、pSV2dhfr ATCC37145、pdBPV-MMTneo ATCC37224、pSV2neo ATCC37149等が、宿主細胞が昆虫細胞の場合は、オートグラフィカル カリホルニカ(Autographica california)核多角体ウイルス(AcNPV)、ボンビックス モリ(Bombyx mori) 核多角体ウイルス(BmNPV)等が好ましい。

本発明のハイブリッド酵素の作製について、Ｇ６ＰＤＨをオリジン酵素として、ＣＲＰ由来ペプチドを外来ペプチドとして、また、大腸菌を宿主細胞として使用した場合を例にとって説明する。

Ｇ６ＰＤＨ遺伝子は、例えば以下に示す方法で得ることができる。即ち、先ずMolecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載の常法に従ってLeuconostoc mesenteroidesの培養物より遠心分離で集菌し、ゲノムＤＮＡを抽出する。尚、以下では特に記載しない限り、Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載された、一般的に行われる周知の技術に従って操作を行う。上記ゲノムＤＮＡを鋳型とし、R.Levyらの文献(J.Biological Chemistry, Vol.266, p13028-, 1991)に示されたＧ６ＰＤＨ遺伝子の配列より、Ｇ６ＰＤＨ遺伝子のＮ－及びＣ－末端配列を含むか、あるいはこれより上流及び下流域の配列を有するオリゴヌクレオチドプライマーを加え、ＤＮＡサーマルサイクラー（パーキンエルマー社）を用いてポリメラーゼ連鎖反応を行い、Ｇ６ＰＤＨ遺伝子を含むＤＮＡ断片を特異的に増幅させる。得られたＤＮＡ断片を常法に従いベクターＤＮＡに組み込むことで、Ｇ６ＰＤＨをコードするＤＮＡを含む、組み換えＤＮＡを得ることができる。

さらに、外来ペプチドをＧ６ＰＤＨ遺伝子に挿入あるいは置換の形で連結するには、いかなる方法でもよく、周知技術である、例えば、「ラボマニュアル遺伝

子工学」（村松正貫　編、第３版、p219～230、丸善）に記載のエキソヌクレアーゼを用いた欠失変異体作製方法やKunkel法、カセット法、PCRを用いる方法のような人工変異導入法、あるいは「ＤＮＡクローニング１」（D.M.GLOVERら編、第２版、p197～228、Takara社）に記載のホスホロチオエート法、ギャップ二本鎖ＤＮＡ法、ＭＨＴプロトコール等を組み合わせて行うことができる。得られたハイブリッド酵素をコードするＤＮＡを常法に従いベクターＤＮＡに組み込むことで、ハイブリッド酵素をコードするＤＮＡを含む組み換えＤＮＡを得ることができる。ここでは、プラスミドに組み込まれたＧ６ＰＤＨ遺伝子中に制限酵素部位を作製し、外来ペプチドを挿入あるいは置換の形で連結する方法について説明する。

即ちG6PDH遺伝子を鋳型とし、ペプチドを挿入したい部分の両側のアミノ酸配列をコードする塩基配列の５’ー側に制限酵素、例えばＢａｍＨＩにより切断され得る部位（ＢａｍＨＩ部位）を付加したプライマーと、これより上流および下流部分をコードする塩基配列のプライマーを組み合わせて、ＰＣＲを行い、ＢａｍＨＩ部位が付加したDNA断片を増幅する。次に、得られた２種類の断片をベクター上で連結することにより、ＢａｍＨＩ部位が挿入されたG6PDH遺伝子が構築できる。制限酵素部位を制限酵素で切断することにより、切断部位と相補的な配列を両端に持つ任意のＤＮＡ断片を挿入することができる。

また、ペプチドを置換挿入するために任意のアミノ酸配列を取り除く場合は、取り除く配列の両側について上記と同様の操作を行うことにより、外来ペプチドが置換の形で連結されたＧ６ＰＤＨ遺伝子を得ることが出来る。

組換えＤＮＡの構築の際には、他の蛋白やペプチドとハイブリッド酵素との融合蛋白として分泌生産させることもでき、さらに、融合蛋白として分泌生産させた後に、適当なプロテアーゼや化学処理による切断を行い、ハイブリッド酵素を得ることもできる。融合させる蛋白としては、例えば、マルトース結合蛋白やグルタチオンＳ－トランスフェラーゼなどが、融合させるペプチドとしてはヒスチジンタグ、ＦＬＡＧタグなどが挙げられる。

組換えＤＮＡ構築の基礎となるベクターとしては、例えば、pBR322（J.G.Sutcliffe,Cold Spring Harbor Symposium,43,77,1979）、pUC18/19（C.Yanisch-Per

ron ,et.al,Gene 33,p102-119,1985）、pBluescriptIISK+（STRATEGENE社）、pMAL-C2（NEW England Biolabs社）、pTrc99A（アマシャム　ファルマシア社）、pKK223-3（アマシャム　ファルマシア社）pET-11（STRATEGENE社）等のプラスミドベクター、λENBL3（STRATEGENE社）、λDASHII（フナコシ社）等のバクテリオファージベクターが挙げられる。

組換えＤＮＡに用いるプロモーターとしては、大腸菌で機能するものであればいずれでもよく、例えば、lacプロモーター、trpプロモーター、Ｔ7プロモーターおよびこれらの誘導体などが挙げられる。また、組換えＤＮＡは、大腸菌で機能するリボソーム結合配列等の開始シグナルや、ターミネーター等を含んでいてもよい。さらに、アンピシリン耐性遺伝子、テトラサイクリン耐性遺伝子などの選択マーカー遺伝子などを含んでいてもよい。

【０００６】

このようにして構築した組換えＤＮＡを用いて大腸菌を形質転換または形質導入し、形質転換体または形質導入体を作製する。大腸菌としては、M103、JA221、HB101、C600、XL1-Blue、JM109などが挙げられる。

組換えＤＮＡを大腸菌へ形質転換または形質導入する方法としては、例えば、Cohenらの方法（Proc.Natl.Acad.Sci.U.S.A.,69,p2110,1972）,Hanahanらの方法（J.Mol.Biol.,166,p557,1983）などが挙げられる。なお、組み換えＤＮＡを有する形質転換体または形質導入体から当該組み換えＤＮＡを得るには、例えばアルカリミニプレップ法等の常法により行えばよい。

本発明のハイブリッド酵素は、上記のごとく調製される組換えＤＮＡの形質転換体または形質導入体を培養することによって製造することができる。使用する培地としては、例えばLuria-Bertani培地（Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)、２×ＹＴ培地(Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)、Ｍ９培地(J.Miller.Exp.Mol.Genet.,Cold Spring Harbor Laboratory,New York,p431,1972)などが挙げられ、培地のpHは５～８であることが望ましい。培養は、通常１４～４２℃、好ましくは２８～３９℃で３～２４時間行うことができ、必要に応じて通気や撹はんを加えてもよい。また、必要により、isopropyl－β-D-1-

thiogalactopyranoside等の発現誘導剤や、アンピシリンやクロラムフェニコール等の抗生物質等を添加してもよい。

本発明のハイブリッド酵素は、上記培養により得られる培養物より以下のようにして取得できる。即ち、ハイブリッド酵素が、培養物のうち培養液中に存在する場合は、得られた培養物をろ過または遠心分離等の常法により、ハイブリッド酵素を含有する培養ろ液または培養上清を得る。一方、ハイブリッド酵素が、培養された形質転換体または形質導入体のペリプラズムまたは細胞内に存在する場合は、培養物をろ過または遠心分離等の常法に付して菌体を集め、適当な緩衝液に懸濁し、例えば超音波やリゾチーム及び凍結融解等の常法で細胞を破壊した後、ろ過または遠心分離等の常法でハイブリッド酵素を含有する粗抽出液を得る。

このようにして得られた本発明のハイブリッド酵素を含有する培養ろ液、培養上清あるいは粗抽出液からハイブリッド酵素を分離、精製するには、自体公知の分離、精製法を適切に組み合わせて実施すればよい。これらの公知の分離、精製法としては、塩析や溶媒沈殿法などの溶解度の差を利用する方法、透析法、限外濾過法、ゲル濾過法、あるいはSDS-ポリアクリルアミドゲル電気泳動法などの主として分子量の差を利用する方法、イオン交換クロマトグラフィーなどの荷電の差を利用する方法、疎水クロマトグラフィーなどの疎水性の差を利用する方法、等電点電気泳動法などの等電点の差を利用する方法、アフィニティークロマトグラフィーなどの特異的親和性を利用する方法などが挙げられる。

得られたハイブリッド酵素の酵素活性に対する抗CRP抗体の効果は、例えば以下のようにして調べることができる。即ち、抗CRP抗体の非存在及び存在下でハイブリッド酵素の酵素活性をそれぞれ測定し、抗CRP抗体結合による活性の変動を調べる。各ハイブリッド酵素液を1%牛アルブミン、3mM塩化マグネシウム、150mM塩化ナトリウムを含有する100mM Tris/HCl緩衝液(pH7.8)で約１U/mlに希釈したもの6μlに、3mM塩化マグネシウムと150mM塩化ナトリウムを含有する100mM Tris/HCl緩衝液(pH7.8；以下、緩衝液Aと省略する)、または抗CRPヤギ抗体を緩衝液Aで100倍希釈した抗体液を150μl添加し、37℃で5分間反応後、10mM グルコース-6-リン酸(G6P)、6mM ニコチンアミドアデニンジヌクレオチド(NAD)を含有する緩衝液A 75μlを添加し、37℃で5間反応させ、波長340nmにおける5分

間の吸光度変化をG6PDH活性として求める。

【０００７】

酵素中に挿入する外来ペプチドは、該ペプチドに結合能を有する物質が結合できる構造を維持しており、該ペプチドの挿入によっても酵素活性が失活しないものであればよいが、Antibodies A Laboratory Manual (Ed Harlow et.al, p76-, Cold spring Harbor Laboratory) 等に記載のように、挿入されるペプチドが抗原性を維持するためには少なくとも６個以上の連続するアミノ酸残基を有する構造である必要がある。また、挿入する外来ペプチドがＣＲＰ由来のペプチドである場合には、配列番号１で示されるＣＲＰの全アミノ酸配列の全部或いは一部が挙げられ、さらには、そのうちの６～５０個の連続したアミノ酸配列を含むもの、例えばＧｌｎ（１）～Ａｓｐ（１６）、Ｇｌｕ（１４）～Ａｌａ（２４）、Ｌｅｕ（２２）～Ｓｅｒ（４５）、Ｔｈｒ（４１）～Ａｓｎ（６１）、Ａｒｇ（４７）～Ｉｌｅ（６３）、Ｌｙｓ（１１４）～Ｌｙｓ（１２１）、Ｇｌｕ（１３０）～Ｇｌｕ（１３８）、Ｉｌｅ（１３４）～Ｇｌｙ（１４８）、Ｇｌｎ（１３７）～Ｌｅｕ（１５２）、Ｇｌｕ（１４７）～Ｌｅｕ（１５２）、Ａｓｐ（３）～Ｓｅｒ（１８）、Ｌｅｕ（１５２）～Ｖａｌ（１６５）、Ｖａｌ（１６５）～Ｇｌｙ（１７８）、Ｌｅｕ（１２１）～Ｓｅｒ（１３２）、Ａｒｇ（１８８）～Ｇｌｕ（１９７）から選ばれる連続したアミノ酸配列が好ましい。中でも配列番号２：Ａｓｐ（３）～Ｓｅｒ（１８）、配列番号３：Ｌｅｕ（１５２）～Ｇｌｙ（１７８）、配列番号４：Ｌｅｕ（１２１）～Ｓｅｒ（１３２）、配列番号５：Ａｒｇ（１８８）～Ｇｌｕ（１９７）に示される配列から選ばれる、少なくとも６個以上の連続したアミノ酸配列を含むものがより好ましい。なお、酵素中に挿入あるいは置換されるペプチド部分には、上記ハイブリッド酵素作成過程で入ることのある制限酵素部位等、目的のペプチド以外の配列が含まれることがあってもよい。

本発明におけるハイブリッド酵素のオリジン酵素に外来ペプチドを挿入する位置は、外来ペプチドの挿入によっても該酵素活性が保持され、挿入された外来ペプチドに該ペプチドと結合能を有する物質が結合すると酵素活性が変化する位置であればよく、酵素表面に露出している部位、活性化に影響し得る部位が候補と

して挙げられる。オリジン酵素としてのＧ６ＰＤＨに外来ペプチドを挿入する好ましい位置についてＣＲＰ由来ペプチドを用いて検討した。即ち、そのＮ末端、３２／３３（３２番目と３３番目の間の意、以下同様）、３７／３８、４８／４９、６６／６７、８７／８８、１３９／１４０、２２６／２２７、２９４／２９５、２９６／２９７、３０２／３０３、３０５～３１０、３６２／３６３、４０９／４１０、Ｃ末端について外来ペプチドの挿入を試みたが表１に示すように、ペプチド挿入時に酵素活性が残存し、抗ＣＲＰ抗体反応時により酵素活性が変動するＣＲＰ測定に有効な部位は、Ｎ末端、１３９／１４０、３０２／３０３、３０５／３０６、３０６／３０７、３０８／３０９、３０９／３１０、３６２／３６３及びＣ末端であることが判明した。

【０００８】

【表１】

挿入部位	ペプチド挿入時の活性	抗体反応時の酵素活性変動
Lys32/Lys33	−	−
Gln37/Lys38	−	−
Gln48/Ala49	+	−
Phe66/Tht67	+	−
Val87/Thr88	−	−
Gly226/Tyr227	−	−
Ala296/Asp297	−	−
Leu305/Asp306	+	+
Asp306/Val307	+	+
Pro308/Ala309	+	+
Ala309/Asp310	+	+
Glu329/Gly310	−	−
Glu362/Gln363	+	+
Lys409/Lys410	−	−
C-末	+	+

酵素のアミノ酸配列の一部が外来ペプチドにより置換されるという意味は、酵素の特定部位のアミノ酸残基またはアミノ酸配列が外来ペプチドのアミノ酸配列に置き換わることをいう。この場合、置換後にもオリジン酵素の酵素活性が保持され、置換された外来ペプチドに該ペプチドと結合能を有する物質が結合すると該酵素活性が変化するようであれば、取り除かれるアミノ酸残基或いはアミノ酸配列に対して置換挿入されるペプチドのアミノ酸の残基数は多くても少なくてもよいが、挿入されるペプチドのアミノ酸残基数と同じ程度が好ましい。また、置換挿入されるペプチドのアミノ酸残基数は、上記の酵素中に挿入する外来ペプチ

ドのそれと同じ程度が好ましい。さらに、外来ペプチドが置換される位置としては、置換後にもオリジン酵素の酵素活性が保持され、置換された外来ペプチドに該ペプチドと結合能を有する物質が結合すると該酵素活性が変化するようであればよく、上記に示したハイブリッド酵素のオリジン酵素に外来ペプチドを挿入し得る位置に準じて選択される。

【０００９】

本発明では、上記のようにして得られたハイブリッド酵素を定性または定量分析に使用する。

本発明のハイブリッド酵素は挿入あるいは置換された外来ペプチドに結合能を有する物質が該酵素に結合すると、該酵素活性がその結合量に応じて変化するものである。従って、本発明のハイブリッド酵素に該結合物質を含む試料を反応させ、酵素活性の変動を測定することにより該結合能を有する物質の存在及びその量を検出することができる。また、本発明のハイブリッド酵素と、該結合能を有する物質とを用いれば、ハイブリッド酵素に挿入あるいは置換された該ペプチドと該ペプチドを含有する物質の該結合能を有する物質への競合により、ハイブリッド酵素に結合する該結合能を有する物質の量が変化することを利用して、該ペプチドを含有する物質の存在またはその量を検出することができる。その方法は（１）被分析物質を含む試料と、本発明のハイブリッド酵素と、本発明のハイブリッド酵素に挿入あるいは置換された外来ペプチドに結合能を有する物質とを接触させ、反応混合物を形成するステップ；（２）前記反応混合物を、出発酵素に対する基質と接触させるステップ；及び（３）反応混合物中に存在する被分析物質の量に従い、ハイブリッド酵素の酵素活性の変化をモニターするステップを含む。ステップ（２）は、反応混合物を定常または平衡状態としてから実施することもできるし、またステップ（１）は順次または同時に実施することができる。（１）において、該結合能を有する物質を含む試料と、ハイブリッド酵素を反応させる操作を行うことにより、該結合能を有する物質の存在及びその量の検出を行うことが出来る。

このように、本発明のハイブリッド酵素含有試薬を用いれば、抗ＣＲＰ抗体の存在または量を直接に測定することが出来、また本発明のハイブリッド酵素に更

に抗ＣＲＰ抗体を含有する試薬を用いて、結合分子たる抗ＣＲＰ抗体への結合の競合によって抗原たるＣＲＰの存在または量を間接的に検出するアッセイを行うことができる。

尚、ハイブリッド酵素の酵素活性の測定は、オリジン酵素の活性測定法に準じて行えばよい。

また、高分子物質をホモジニアスな系で測定するための、外来ペプチドが挿入されても同様の酵素活性を有し、且つ外来ペプチド部分に結合能を有する物質が結合した場合、当該酵素活性が変化し得る、G6PDH含有ハイブリッド酵素を作成するために用いられるG6PDHとしては、配列番号６に示したアミノ酸配列、或いは当該配列のうち、１もしくは複数のアミノ酸が欠失、置換あるいは付加された配列を有するものや、由来の異なるＧ６ＰＤＨであっても、Ｇ６ＰＤＨ活性を有するもの等が含まれる。また、外来ペプチドが挿入される位置については上記で示した位置が好ましいものとして挙げられる。

また、外来ペプチドとしては先に挙げた全てのものが挙げられ、このうちＣＲＰについては、上記のようなペプチドを用いることが好ましい。

また、Ｇ６ＰＤＨ以外の酵素を用いて作成されるＣＲＰ由来のペプチドをその一部として含む外来ペプチドが挿入または置換されたハイブリッド酵素も上で述べたものと同様な方法により作成される。また、外来ペプチドが挿入または置換される位置についても同様にして適宜選択すればよい。尚、酵素としてβ－ガラクトシダーゼを用いる場合は、FEBS Letters434(1998)23-27、FEBS Letters 438(1998)267-271の記載に準じて、アルカリホスファターゼを用いる場合にはProc. Natl.Acad.Sci.USA92(1995)に記載の方法に準じて選択してもよい。

このようにして得られた、外来ペプチドが挿入されたハイブリッド酵素を用いれば、当該外来ペプチドに結合能を有する物質の存在またはその量を直接に測定することができ、また、当該ハイブリッド酵素と当該外来ペプチドに対する抗体とを組み合わせて用いれば、当該外来ペプチドを含む高分子物質の存在又は量を間接的に検出するアッセイを行うことができる。尚、これらアッセイは、上で述べたＣＲＰ或いは抗ＣＲＰ抗体の測定操作に準じて行えばよい。更に、ハイブリッド酵素を用いることによりホモジニアスな系で高分子物質を高感度に測定する

ことが出来、この方法は汎用の自動分析装置に応用することもできる。

以下に本発明を更に詳しく説明するために実施例を挙げるが、本発明はこれに限定されるものではない。

【００１０】

【実施例】

実施例１

G6PDH遺伝子を含むプラスミドの構築

Leuconostoc mesenteroidesをLACTOBACILLI MRS BROTH (DIFCO社) 5ml に接種して、26℃で16時間振とう培養し培養物を得た後、この培養物を4℃、6000rpmで10分間遠心分離することにより集菌して菌体を得た。1mM エチレンジアミン四酢酸(EDTA)を含有する10mMトリス（ヒドロキシメチル）アミノメタン(Tris/HCl)（以下、TEと省略する。）緩衝液に懸濁し、終濃度300u/mlのアクロモペプチダーゼを添加して37℃、2時間静置した後、終濃度0.5％のSDSと終濃度100μg/mlのProteinaseKを添加して、さらに37℃、2時間静置して溶菌を行った。Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載の定法に従って、G6PDH（以下、G6PDHと省略する。）遺伝子の供与体として、Leuconostoc mesenteroidesのゲノムDNAを抽出した。なお、以下では特に記載しない限り、Molecular Cloning(J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載された、一般的に行われる周知の技術に従って操作を行った。次に、G6PDH遺伝子を得るために、以下の手順でポリメラーゼ連鎖反応（以下、PCRと省略する。）を行った。鋳型DNAとして上記ゲノムDNAを10ng、R.Levyらの文献(J.Biological Chemistry, Vol.266, p13028-, 1991)に示されたG6PDH遺伝子のN-及びC-末端配列を含む、各々配列番号７、8に記載のオリゴヌクレオチドプライマーを各0.1 nmol加え、DNAサーマルサイクラー（パーキン・エルマー社）を用い、94℃で30秒、65℃で30秒、72℃で4分のサイクルを25回繰り返して反応を行った。その結果、G6PDH遺伝子を含む約1.5 kbpのDNA断片が特異的に増幅された。得られたDNA断片をクローニングベクターpBluescriptＩＩ KS+（Stratagene社）のEcoRＶ部位へ連結して、プラスミドpBSWGを構築した。

次いで、クローニングベクターpUC18を EcoRIとSalIで消化した後、その末端を平滑化したものに得られたＤＮＡ断片を連結し、Ｇ６ＰＤＨ遺伝子の発現が可能なプラスミドでpUCGを構築した。さらに、Kunkel法に従い、配列番号9に示したオリゴヌクレオチドプライマーとMutan-K（宝酒造社）を使用して、G6PDH遺伝子のN-末端から750番目の塩基シトシンをチミンに変異させ、アミノ酸配列を変えることなく、N-末端以外には制限酵素Nco I認識配列を有さないG6PDH遺伝子を含む、プラスミドpBSMGを構築した。これを制限酵素NcoI及びPst Iで消化して、約1.5kbpのG6PDH遺伝子を回収し、プラスミドpUCGを制限酵素Nco I及びPst Iで消化した約２．７kbpのDNA断片と連結して、プラスミドpUCMGを構築した。

【００１１】

実施例２

G6PDHのPro308/Ala309間にヒトCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例1のプラスミドpUCMGを鋳型とし、配列番号7及び、Pro308上流のアンチセンス鎖配列の5’-側に制限酵素BamH I認識配列を付加した配列番号10に記載のオリゴヌクレオチドプライマーを用いてPCRを行い、下流部位に制限酵素BamH I認識配列が付加されたG6PDH遺伝子N末端からPro308までを含む約0.9kbpのDNA断片を得た。同様に、Ala309下流のセンス鎖配列の5’-側に制限酵素BamH I認識配列を付加した配列番号11に記載のオリゴヌクレオチドプライマーと、G6PDH遺伝子C-末端のアンチセンス鎖配列を含む配列番号12に記載のオリゴヌクレオチドプライマーを用いて、上流部位に制限酵素BamH I認識配列が付加された、G6PDH遺伝子のAla309からC末端を含む約0.6kbpのDNA断片を得た。N末端側断片を制限酵素BamH IとNco I、C末端側断片を、制限酵素BamH IとPst Iでそれぞれ消化した後、プラスミドpUCMGを制限酵素NcoＩとPstＩ部位で消化して得られる約2.7kbpのDNA断片と連結することで、G6PDH遺伝子のPro308/Ala309間にのみ制限酵素BamＨＩ配列を有する組み換え体　pUCMG308Bを構築した。これを制限酵素BamＨＩで開裂させ、配列番号２に記載のアミノ酸配列をコードするDNAを含み且つそれらが相補である合成ヌクレオチド(配列番号13と配列番号14との組合せ）を連結しp

UCMG308C1を、配列番号３に記載のアミノ酸配列の一部をコードするDNAを含み且つそれらが相補な合成ヌクレオチドである配列番号15と配列番号16との組合せ又は配列番号21と配列番号22との組合せを連結しpUCMG308C2及びpUCMG308C13を、配列番号４に記載のアミノ酸配列をコードするDNAを含み且つそれらが相補である合成ヌクレオチド(配列番号17と配列番号18との組合せ）を連結しpUCMG308C3を、配列番号5に記載のアミノ酸配列をコードするDNAを含み且つそれらが相補である合成ヌクレオチド(配列番号19と配列番号20との組合せ）を連結しpUCMG308C5を、夫々構築した。尚、各合成ヌクレオチドは次のような相補構造から成っている。

【化１】

5'-gatccgacatgtcgaggaaggcttttgtgtttcccaaagagtcggatacttccg -3'　配列番号13

3'- gctgtacagctccttccgaaaacacaaagggtttctcagcctatgaaggcctag-5'　配列番号14

5'-gatccgtgctgtcaccagatgagattaacaccatctatcttggcgggg -3'　配列番号15

3'- gcacgacagtggtctactctaattgtggtagatagaaccgccccctag-5'　配列番号16

5'-gatccctgaagaagggatacactgtgggggcagaagcaagcg -3'　配列番号17

3'- ggacttcttccctatgtgacacccccgtcttcgttcgcctag-5'　配列番号18

5'-gatcccgggcactgaagtatgaagtgcaaggcgaag -3'　配列番号19

3'- ggcccgtgacttcatacttcacgttccgcttcctag-5'　配列番号20

5'-gatcctagtgggagacattggaaatgtgaacatgtgggactttgtgg-3'　配列番号21

3'- gatcaccctctgtaacctttacacttgtacaccctgaaacaccctag-5'　配列番号22

【００１２】

実施例３

融合酵素G308C1、G308C2、 G308C3、 G308C5及びG308C13の発現及び抽出

実施例2の組み換えDNA　pUCMG308C1、 pUCMG308C3、pUCMG308C5、 pUCMG308C2及びpUCMG308C13　を大腸菌XL1-Blueに形質転換した。Levyら（Protein Science,1,329-,1992）の方法に従って、形質転換した大腸菌を37℃16時間LB培地（DIF

CO社）で振とう培養して得た培養液を、終濃度が2%となるようにLB培地に植菌し、37℃5時間振とう培養後、IPTG（Isopropyl-β-D-thiogalactopyranoside）を終濃度0.5mMで添加して発現を誘導し、37℃16時間培養した後、4℃、6000rpmで10分間遠心分離することにより菌体を得た。これを 8% sucrose、0.1% Triton-X、50mM EDTAを含有する10mM Tris/HCl緩衝液に懸濁後、終濃度33mg/mlのリゾチームを添加して、37℃、30分間静置した後、15000rpm、20分間の遠心操作で、不溶物を取り除いて融合酵素G308C1、G308C2、 G308C3、 G308C5及びG308C13液をそれぞれ得た。なお、対照として、プラスミドpUCMGについても同様に行い、野生型酵素液を得た。

【００１３】

実施例４

融合酵素G308C1、G308C2、G308C3、G308C5及びG308C13の酵素活性に対する抗CRP抗体の効果

実施例3で得た融合酵素液の活性を、抗CRP抗体の非存在及び存在下でそれぞれ測定し、抗CRP抗体結合による酵素活性の変動を調べた。各融合酵素液を1%牛アルブミン、3mM塩化マグネシウム、150mM塩化ナトリウムを含有する100mM Tris/HCl緩衝液(pH7.8)で400倍希釈したもの6μlに、3mM塩化マグネシウムと150mM塩化ナトリウムを含有する100mM Tris/HCl緩衝液(pH7.8；以下、緩衝液Aと省略する)、または抗CRPヤギ抗体を緩衝液Aで100倍希釈した抗体液を150μl添加し、37℃で5分間反応後、10mM グルコース-6-リン酸(G6P)、6mM ニコチンアミドアデニンジヌクレオチド(NAD)を含有する緩衝液A 75μlを添加し、37℃で5間反応させ、波長340nmにおける5分間の吸光度変化をG6PDH活性として求めた。抗CRP抗体非存在時の酵素活性に対する、抗体存在時の活性を活性比として表２に示した。その結果、野生型G6PDHは、抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素G308C1、G308C2、G308C3、G308C5及びG308C13では、抗CRP抗体存在時の酵素活性が、非存在時よりも低くなることが判る。すなわち、G6PDHの Pro308/Ala309間に配列番号2、3、4、5に記載のCRPﾍﾟﾌﾟﾁﾄﾞ或いはその一部を挿入した融合酵素では、抗CRP抗体の結合により、その酵素活性が阻害されることが判る。

【表２】

	活性比
野生型	101%
融合酵素	
G308C1	86%
G308C2	29%
G308C3	36%
G308C5	88%
G308C13	39%

$$\text{活性比(\%)} = \frac{\text{抗体存在時のG6PDH活性}}{\text{抗体非存在時のG6PDH活性}} \times 100$$

実施例５

融合酵素G308C1を用いた抗CRP抗体の測定

抗CRP抗体量依存的な融合酵素G308C1活性の変動を調べた。抗CRPモノクローナル抗体を緩衝液Aで100倍、1000倍、10000倍、100000倍希釈した各溶液あるいは緩衝液A　50μlに、緩衝液Aで3300倍希釈した融合酵素G308C1液を100μl添加し、37℃で5分間反応後、10mM　G6P、6mM　NADを含有する緩衝液A　75μlを添加して、37℃で5分間反応させて、波長340nmにおける5分間の吸光度変化をG6PDH活性として求めた結果を図1に示す。その結果から判るように、抗CRP抗体量の減少に伴い酵素活性が増加する現象が見られた。すなわち、G6PDHの　Pro308/Ala309間に配列番号2に記載のCRPペプチドを挿入した融合酵素を用いて、抗CRP抗体が測定できることが示された。

【００１４】

実施例６

融合酵素G308C1を用いたCRPの測定

抗CRP抗体の結合により阻害される融合酵素活性の、CRP濃度による回復を測定した。各種濃度（0、10、20、40 mg/dl）のCRP溶液6μlに、緩衝液Aで3300倍希釈した融合酵素G308C1液を100μl添加し、37℃で3分間反応後、緩衝液Aで10000倍希釈した抗CRPモノクローナル抗体液50μlを添加した。さらに37℃で3分間反応させ、10mM　G6P、6mM　NADを含有する緩衝液A　75μlを添加して、37℃で5分

間反応させて、波長340nmにおける5分間の吸光度変化をG6PDH活性として求めた。図２に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHの　Pro308/Ala309間に配列番号2に記載のCRPﾍﾟﾌﾟﾁﾄﾞを挿入した融合酵素を用いて、CRPが測定できることが示された。

実施例７

融合酵素G308C2、G308C3、G308C5を用いたCRPの測定

実施例６と同様の方法により、CRPの測定を試みた。なお、融合酵素G308C2、G308C3、G308C5液の希釈は各々5000倍、15000倍、2000倍、抗CRPヤギ抗体の希釈倍率は全て8倍で実施した。その結果、図3、4、５に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのPro308/Ala309間に配列番号３，4、5に記載のCRPﾍﾟﾌﾟﾁﾄﾞを挿入した融合酵素を用いて、CRPが測定できることが示された。

【００１５】

実施例８

G6PDHのAsp306/Val307間に、CRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例2と同様の操作により、配列番号7と23、12と24に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のAsp306/Val307間にのみ制限酵素BamH I 配列を有する組み換え体　pUCMG306Bを構築し、配列番号13、14に記載の合成オリゴヌクレオチドを連結して、組み換えDNA　pUCMG306C1を構築した。

実施例９

融合酵素G306C1を用いたCRPの測定

実施例3と同様の操作により、融合酵素G306C1液を得た。次いで、実施例6と同様の方法により、CRPの測定を試みた。なお、融合酵素液の希釈は500倍、抗CRPモノクローナル抗体の希釈倍率は3200倍で実施した。その結果、図６に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのAsp306/Val307間に配列番号2に記載のCRPﾍﾟﾌﾟﾁﾄﾞを挿入した融合酵素を用いて、CRPが測定できることが示された。

【００１６】

実施例10

G6PDHのAla309/Asp310間にCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例2と同様の操作により、配列番号7と25、12と26に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のAla309/Asp310間にのみ制限酵素BamHＩ配列を有する組み換え体　pUCMG309Bを構築し、配列番号13、14に記載の合成オリゴヌクレオチドを連結して、組み換えDNA　pUCMG309C1を構築した。

実施例11

融合酵素G309C1を用いたCRPの測定

実施例3と同様の操作により、融合酵素G309C1液を得た。次いで、実施例6と同様の方法により、CRPの測定を試みた。なお、融合酵素液の希釈は2500倍、抗CRPモノクローナル抗体の希釈倍率は10000倍で実施した。その結果、図７に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのAla309/Asp310間に配列番号2に記載のCRPﾍﾟﾌﾟﾁﾄﾞを挿入した融合酵素を用いて、CRPが測定できることが示された。

【００１７】

実施例12

G6PDHのＣ末端にCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

Kunkel法に従い、プラスミドpBSMG、配列番号27に示したオリゴヌクレオチドプライマー及び、Mutan-K（宝酒造社）を使用して、G6PDH遺伝子のC-末端に制限酵素BamHＩ配列を付加したプラスミドpBSMGCBを得た。次いで、実施例1、2と同様の操作により、C-末端にのみ制限酵素BamHＩ配列を有する組み換え体　pUCMGCBを構築し、配列番号13に記載の合成オリゴヌクレオチドを連結して、組み換えDNA　pUCMGCC1を構築した。

実施例13

融合酵素GCC1の酵素活性に対する抗CRP抗体の効果

実施例3と同様の操作により、融合酵素GCC1液を得た。次いで、実施例4と同様の操作により、抗CRP抗体が融合酵素GCC1に結合した場合の効果を調べた。抗CRP抗体非存在時の酵素活性に対する、抗体存在時の活性を活性比として表３に示した。その結果、野生型G6PDHは、抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素GCC1は、抗CRP抗体存在時の酵素活性が、非存在時よりも低くなることが示された。すなわち、G6PDHのC末端に配列番号2に記載のCRPﾍﾟﾌﾟﾁﾄﾞを連結した融合酵素では、抗CRP抗体の結合により、その酵素活性が阻害されることが示された。

【表３】

	活性比
野生型	101%
融合酵素GCC1	95%

$$\text{活性比}(\%) = \frac{\text{抗体存在時のG6PDH活性}}{\text{抗体非存在時のG6PDH活性}} \times 100$$

【００１８】

実施例14

G6PDHのGlu362/Gln363間にCRP由来ペプチドを連結した融合酵素をコードする組み換えDNAの構築

実施例2と同様の操作により、配列番号7と28、12と29に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のGlu362/Gln363間にのみ制限酵素BamHＩ配列を有する組み換え体　pUCMG362Bを構築し、配列番号13、14に記載の合成オリゴヌクレオチドを連結して、組み換えDNA　pUCMG362C1を構築した。

実施例15

融合酵素G362C1を用いた抗CRP抗体の測定

実施例3と同様の操作により、融合酵素G362C1液を調製し、次いで、実施例5と

同様の方法により抗ＣＲＰ抗体の量による融合酵素G362C1活性の変動を調べた。その結果、図８に示したように、抗CRP抗体量の増加に伴い酵素活性が増加する現象が見られた。すなわち、G6PDHのGlu362/Gln363間に配列番号2に記載のCRPﾍﾟﾌﾟﾁﾄﾞを挿入した融合酵素を用いて、抗CRP抗体が測定できることが示された。

【００１９】

実施例16

配列番号２に記載のCRP由来ペプチドの一部をG6PDHに挿入した融合酵素

実施例8で構築したG6PDH遺伝子のAsp306/Val307間にのみ制限酵素BamH Ｉ配列を有する組み換え体　pUCMG306Bに、実施例2と同様の操作により、配列番号２に記載のアミノ酸配列の一部をコードするDNAを含む配列番号32と33、あるいは、配列番号34と35の合成ヌクレオチドをそれぞれ連結し、組み換えDNA　pUCMG306C15及びpUCMG306C18を夫々構築した。同様に、実施例2の組み換え体　pUCMG308Bを用い、組み換えDNA pUCMG308C15及びpUCMG308C18を夫々構築した。これらを用い、実施例3と同様の操作によって融合酵素G306C15、G306C18、G308C15、G308C18を得た。これらの融合酵素と実施例9及び実施例3で得た融合酵素G306C1及びG308C1について、実施例4と同様の操作により、抗CRP抗体が各融合酵素に結合した場合の効果を調べ、抗CRP抗体非存在時の酵素活性に対する、抗体存在時（抗CRPモノクローナル抗体　1000倍希釈）の活性を活性比として表４に示した。次に、融合酵素G306C1、G306C15及びG306C18を用いて、CRP測定感度の比較を行った。各種濃度（0、5、10、20、40 mg/dl）のCRP溶液10μlに、緩衝液Aで希釈した各融合酵素と抗CRPモノクローナル抗体との混合液を250μl添加し、37℃で5分間反応後、10mM　G6PDH、6mM　NADを含有する緩衝液A　125μlを添加して、さらに37℃で5分間反応させて、波長340nmにおける5分間の吸光度変化をG6PDH活性として求めた。なお、各融合酵素の希釈倍率はG306C1、G306C15、G306C18それぞれ10400倍、6240倍、58500倍、抗CRPモノクローナル抗体は全て15600倍希釈で実施した。結果を図9に示す。以上示した結果より、ペプチドを挿入する酵素部位の選択、且つ／又は挿入するペプチド長の選択により、測定感度等を調節することができる可能性が示された。

【表４】

	活性比
野生型	100.4%
融合酵素	
G306C1	53.1%
G306C15	54.0%
G306C18	16.2%
G308C1	78.9%
G308C15	89.8%
G308C18	94.6%

尚、各融合酵素に挿入したCRPペプチド配列を以下に示す。

G306C1、G308C1；

Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp Thr Ser

G306C15、G308C15；

Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser

G306C18、G308C18；

Arg Lys Ala Phe Val Phe Pro Lys Glu Ser

【００２０】

実施例17

G6PDHのN末端、Asp294/Ser295間及びLeu302/Glu303間にCRP由来ペプチドを挿入した融合酵素

Kunkel法に従い、プラスミドpBSMG、配列番号36に示したオリゴヌクレオチドプライマー及び、Mutan-K（宝酒造社）を使用して、G6PDH遺伝子のN-末端に制限酵素BamHＩ配列を付加したプラスミドpBSMGNBを得た。次いで、実施例1、2と同様の操作により、N-末端にのみ制限酵素BamHＩ配列を有する組み換え体　pUCMGNBを構築した。また、実施例2と同様の操作により、配列番号7と37、12と38に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のAsp294/Ser295間にのみ制限酵素BamHＩ配列を有する組み換え体pUCMG294Bを、配列番

号7と39、12と40に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のLeu302/Glu303間にのみ制限酵素BamHＩ配列を有する組み換え体pUCMG302Bを構築した。次いでこれらに配列番号34、35に記載の合成オリゴヌクレオチドを連結して、組み換えDNA　pUCMGNC18、pUCMG294C18及びpUCMG302C18を構築した。実施例3と同様の操作により、各融合酵素液GNC18、G294C18、G302C18を得た。実施例4と同様の操作により、抗CRP抗体が各融合酵素に結合した場合の効果を調べ、抗CRP抗体非存在時の酵素活性に対する、抗体存在時（抗CRPモノクローナル抗体　1000倍希釈）の活性を活性比として表5に示した。その結果、野生型G6PDHは、抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素GNC18、G294C18、G302C18は、抗CRP抗体存在時の酵素活性が、非存在時よりも低くなることが示された。すなわち、G6PDHのN末端、Asp294/Ser295間、Leu302/Glu303間にCRPペプチドを挿入した融合酵素は、いずれも抗CRP抗体の結合によりその酵素活性が阻害されることが示された。

【表５】

	活性比
野生型	100.3%
融合酵素 GNC18	90.2%
G294C18	51.4%
G302C18	57.5%

【００２１】

実施例18

G6PDHのAsp306/Val307間とC末端の２カ所にCRP由来ペプチドを挿入した融合酵素

実施例12で構築した組み換え体　pUCMGCBに、実施例2と同様の操作により、配列番号32、33に記載の合成オリゴヌクレオチドを連結して、組み換えDNA　pUCMGCC15を構築し、これを制限酵素Bpu1102I及びPstＩで消化して、約0.5kbpの断片を回収した。また、実施例16で構築した組み換えDNA　pUCMG306C18を制限酵素NcoＩ及びBpu1102Ｉで消化して、約1.0kbpの断片を回収した。さらに実施例1で構

築したプラスミドpUCMGを制限酵素NcoＩ及びPstＩで消化して約2.7kbpのDNA断片を回収した。これら３つの回収した断片を連結することにより、G6PDH遺伝子の２カ所にCRP由来ペプチドをコードする合成オリゴヌクレオチドが挿入された組み換えDNA　pUCMG306C18+CC15を構築した。実施例3と同様の操作により、融合酵素液G306C18+CC15を得た。実施例4と同様の操作により、抗CRP抗体が融合酵素に結合した場合の効果を調べ、抗CRP抗体非存在時の酵素活性に対する、抗体存在時（抗CRPモノクローナル抗体　1000倍希釈）の活性を活性比として表6に示した。その結果、野生型G6PDHは、抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素G306C18+CC15は、抗CRP抗体存在時の酵素活性が、非存在時よりも低くなることが示された。すなわち、G6PDHのAsp306/Val307間とC末端の２カ所に配列番号２に記載のCRPペプチドの一部を挿入した融合酵素は、抗CRP抗体の結合によりその酵素活性が阻害されることが示された。

【表６】

	活性比
野生型	99.9%
融合酵素 G306C18+CC15	15.2%

【００２２】

実施例19

G6PDHのVal307からAla309のかわりにヒトCRP由来ペプチドを置換した融合酵素

実施例8で作製した、下流部位に制限酵素BamHＩ認識配列が付加されたG6PDH遺伝子N末端からAsp306までを含む約0.9kbpのDNA断片、及び実施例10で作製した、上流部位に制限酵素BamHＩ認識配列が付加されたG6PDH遺伝子のAsp310からC末端を含む約0.6kbpのDNA断片を組み合わせ、実施例2と同様の操作により、G6PDH遺伝子のVal307からAla309のかわりに制限酵素BamHＩ配列を置換した組み換え体pUCMG306d3Bを構築し、配列番号13、14に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMG306d3C1を構築した。実施例3と同様の操作により、融合酵素G306d3C1液を得た。次いで、実施例6と同様の方法により、CRPの測定を行っ

た。なお、融合酵素液の希釈は5000倍、抗CRPモノクローナル抗体の希釈倍率は10000倍で実施した。その結果、図10に示したように、CRP濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのVal307からAla309のかわりに、配列番号2に記載のCRPペプチドを置換した融合酵素を用いて、CRPが測定できることが示された。

【００２３】

実施例20

G6PDHのAsp306をTyrに置換し、Tyr306/Val307間に、制限酵素認識配列を付加することなくCRP由来ペプチドを連結した融合酵素

実施例2と同様の操作により、配列番号7と41、12と42に示したオリゴヌクレオチドプライマーの組み合わせを用いて、G6PDH遺伝子のAsp306をTyrに置換することにより、Tyr306/Val307をコードする配列を制限酵素Eco105 I配列とした組み換え体pUCMG306Eを構築し、配列番号43、44に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMG306EC18を構築した。実施例3と同様の操作により、融合酵素G306EC18液を得た。次いで、実施例4と同様の操作により、抗CRP抗体が融合酵素に結合した場合の効果を調べ、抗CRP抗体非存在時の酵素活性に対する、抗体存在時（抗CRPモノクローナル抗体　1000倍希釈）の活性を活性比として表7に示した。その結果、野生型G6PDHは、抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素G306EC18は、抗CRP抗体存在時の酵素活性が、非存在時よりも低くなることが示された。すなわち、G6PDHのAsp306をTyrに置換し、Tyr306/Val307間に配列番号2に記載のCRPペプチドの一部を挿入した融合酵素は、抗CRP抗体の結合によりその酵素活性が阻害されることが示された。

【表７】

	活性比
野生型	１０１．０％
融合酵素 Ｇ３０６ＥＣ１８	８５．０％

【００２４】

実施例21

G6PDHのAsp306/Val307間に、B型肝炎ウイルスpreS2抗原由来ペプチドを挿入した融合酵素

実施例8で作製したpUCMG306Bに、配列番号45に記載のpreS2抗原（S.Usuda, et.al, J.Virol.Methods 80 (1999) 97-112）のうち一部の配列（配列番号46）をコードする、配列番号47、48に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMG306H1を構築した。実施例3と同様の操作により、融合酵素G306H1液を得た。次いで、実施例5と同様の方法により抗preS2抗体の量による融合酵素G306H1活性の変動を調べた。その結果、図11に示したように、抗preS2抗体量の増加に伴い酵素活性が低下する現象が見られた。すなわち、G6PDHのAsp306/Val307間に配列番号46に記載のpreS2ペプチドを挿入した融合酵素を用いて、抗preS2抗体が測定できることが示された。

【００２５】

実施例22

G6PDHのAsp306/Val307間に、副甲状腺ホルモン（PTH）由来ペプチドを挿入した融合酵素

実施例8で作製したpUCMG306Bに、配列番号49に記載のPTH（J.H.Habener, et.al, Metabolic Bone Disease, 2nd Edition, WB Saunders, Philadelphia (1999) 69）のうち一部の配列（配列番号50）をコードする、配列番号51、52に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCMG306P1を構築した。実施例3と同様の操作により、融合酵素G306P1液を得た。次いで、実施例6と同様の方法により、PTHの測定を行った。なお、融合酵素液の希釈は2000倍、抗PTHモノクローナル抗体の希釈倍率は8000倍で実施した。その結果、図12に示したように、PTH濃度の増加に伴い活性が回復する現象が見られた。すなわち、G6PDHのAsp306/Val307間に配列番号50に記載のPTHペプチドを挿入した融合酵素を用いて、PTHが測定できることが示された。

【００２６】

実施例23

βーガラクトシダーゼ遺伝子を含むプラスミドの構築

大腸菌（Escherichia coli ATCC 25922）をLB培地（DIFCO社製）3mlに接種して、37℃で16時間振とう培養し培養物を得た後、この培養物を4℃、6000rpmで10分間遠心分離することにより集菌して菌体を得た。得られた菌体より、Molecular Cloning (J.Sambrook et.al, 2nd Edition, Cold Spring Harbor Laboratory)等に記載の定法に従って、大腸菌のゲノムDNAを抽出した。次に、βーガラクトシダーゼ遺伝子を得るために、以下の手順でPCRを行った。鋳型DNAとして上記ゲノムDNAを10 ng、A.Kalninsらの文献（EMBO J. 2 (1983) 593-)に示された、βーガラクトシダーゼ遺伝子のN-及びC-末端配列を含む、各々配列番号53、54に記載のオリゴヌクレオチドプライマーを各0.1 nmol加え、DNAサーマルサイクラー（パーキン・エルマー社）を用い、94℃で30秒、60℃で1分、72℃で5分のサイクルを25回繰り返すことによって行った。その結果、βーガラクトシダーゼ遺伝子を含む約3.1 kbpのDNA断片が特異的に増幅された。一方、クローニングベクターpUC19をEcoR IとHindIIIで消化し、クレノーフラグメントで末端を平滑化したものに、PCRで得られたDNA断片を連結し、βーガラクトシダーゼをコードする遺伝子が発現可能な状態でクローニングされたプラスミドpUCBを得た。

【００２７】

実施例24

βーガラクトシダーゼのVal796/Ser797間にCRP由来ペプチドを挿入した融合酵素をコードする組み換えDNAの構築

実施例2と同様の方法により、実施例23で得られたプラスミドpUCBを鋳型とし、配列番号53と55、及び54と56に記載のオリゴヌクレオチドプライマーを用いたPCRを行って、β-ガラクトシダーゼ遺伝子Val796下流部位及びSer797上流部位に制限酵素BamH I 認識配列が付加された各々約2.4kbp、0.7kbpのDNA断片を得た。N末端側断片を制限酵素Sac IとBamH I、C末端側断片を制限酵素BamH IとNde Iでそれぞれ消化して得た各々約0.4kbp、0.6kbpのDNA断片と、プラスミドpUCBを制限酵素Sac IとNde Iで消化して得られる約4.8kbpのDNA断片とを連結し、βーガ

ラクトシダーゼ遺伝子のVal796/Ser797間にのみ制限酵素BamH I 配列を有する組み換え体pUCB796Bを構築した。これに配列番号13、14に記載の合成オリゴヌクレオチドを連結して、組み換えDNA pUCB796C1を構築した。

実施例25

融合酵素B796C1の酵素活性に対する抗CRP抗体の効果

組み換え体としてpUCB796C1を用いた以外は実施例3と同様の操作により、融合酵素B796C1液を得た。次いで、Villaverdeらの方法（FEBS Letters 434 (1998) 23-）に従い、融合酵素液のβ－ガラクトシダーゼ活性を、抗CRP抗体の非存在及び存在下でそれぞれ測定し、抗CRP抗体結合による酵素活性の変動を調べた。融合酵素液を0.1M 2-メルカプトエタノール、1.0mM塩化マグネシウムを含む100mMリン酸緩衝液(pH7.3；以下、緩衝液Bと省略する)で50倍希釈したもの6μlに、緩衝液B、あるいは抗CRPモノクローナル抗体を緩衝液Bで7500倍希釈した抗体液を150μl添加し、37℃で5分間反応させた。次に、17μM o-ニトロフェニル-β-D-ガラクトピラノシドを含む緩衝液B（以下、ONPG液と省略する）24μlを添加し、37℃で5分間反応させ、波長405nmにおける5分間の吸光度変化をβ－ガラクトシダーゼ活性として求めた。抗CRP抗体非存在時の酵素活性に対する、抗体存在時の活性を活性比とした。その結果、表8に示したように、野生型β－ガラクトシダーゼは抗CRP抗体の非存在時と存在時とで活性に差が見られないのに対し、融合酵素B796C1では抗CRP抗体存在時の酵素活性が非存在時よりも高くなることが明らかになった。すなわち、β－ガラクトシダーゼのVal796/Ser797間に配列番号2に記載のCRPペプチドを挿入した融合酵素では、抗CRP抗体の結合により、その酵素活性が増幅されることが確認された。

【表８】

	活性比
野 生 型	１００．２％
融 合 酵 素 Ｂ７９６Ｃ１	１７０．５％

【００２８】

実施例26

融合酵素B796C1を用いた抗CRP抗体の測定

抗CRP抗体量依存的な融合酵素B796C1活性の変動を調べた。抗CRPモノクローナル抗体を緩衝液Bで1000倍、10000倍、100000倍希釈した各溶液あるいは緩衝液B 50μlに、緩衝液Bで1250倍希釈した融合酵素B796C1液を100μl添加し、37℃で5分間反応させた。次に、ONPG液24μlを添加し、37℃で5分間反応させ、波長405nmにおける5分間の吸光度変化をβ－ガラクトシダーゼ活性として求めた。その結果、図13に示したように、抗CRP抗体量の増加に伴い酵素活性が増加する現象が見られた。すなわち、β－ガラクトシダーゼのVal796/Ser797間に配列番号2に記載のCRPペプチドを挿入した融合酵素を用いて、抗CRP抗体が測定できることが示された。

実施例27

融合酵素B796C1を用いたCRPの測定

融合酵素B796C1と抗CRP抗体を用いてCRPの測定を行った。各種濃度（0、10、20、40 mg/dl）のCRP溶液6μlに、緩衝液Bで1250倍希釈した融合酵素B795C1液を100μl添加し、37℃で3分間反応後、緩衝液Bで7500倍希釈した抗CRPモノクローナル抗体液50μlを添加し、さらに37℃で3分間反応させた。次に、ONPG液24μlを添加し、37℃で5分間反応させ、波長405nmにおける5分間の吸光度変化をβ－ガラクトシダーゼ活性として求めた。その結果、図14に示したように、抗CRP抗体の結合により増加した酵素活性が、CRP濃度の増加に伴い回復する現象が見られた。すなわち、β－ガラクトシダーゼのVal796/Ser797間に配列番号2に記載のCRPペプチドを挿入した融合酵素を用いて、CRPが測定できることが示された。

【００２９】

【発明の効果】

本発明の外来ペプチドが挿入されたハイブリッド酵素を用いれば、当該外来ペプチドに結合能を有する物質の存在又はその量を直接に測定することができ、また、当該ハイブリッド酵素と当該外来ペプチドに結合能を有する物質とを組み合わ

せて用いれば、当該外来ペプチドを含む高分子物質の存在または量を間接的に検出・測定することが出来、さらに本発明のハイブリッド酵素を用いることで、ホモジニアスな比色法で試料中の微量なＣＲＰ測定が可能となり、組織中の炎症の早期発見、ひいては早期の病気の診断が可能となり、また各種高分子物質をホモジニアスな系で容易に測定することができる。

【００３０】

【配列表】

SEQUENCE LISTING

<110> WAKO PURE CHEMICAL INDUSTRIES, LTD.

<120> Hybrid Enzymes and Use Thereof

<130> WJ018

<140>

<141>

<160> 56

<170> PatentIn Ver. 2.1

<210> 1

<211> 206

<212> PRT

<213> Human

<400> 1

Gln Thr Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp
1 5 10 15

Thr Ser Tyr Val Ser Leu Lys Ala Pro Leu Thr Lys Pro Leu Lys Ala
20 25 30

Phe Thr Val Cys Leu His Phe Tyr Thr Glu Leu Ser Ser Thr Arg Gly
35 40 45

Tyr Ser Ile Phe Ser Tyr Ala Thr Lys Arg Gln Asp Asn Glu Ile Leu
50 55 60

Ile Phe Trp Ser Lys Asp Ile Gly Tyr Ser Phe Thr Val Gly Gly Ser
65 70 75 80

Glu Ile Leu Phe Glu Val Pro Glu Val Thr Val Ala Pro Val His Ile
85 90 95

Cys Thr Ser Trp Glu Ser Ala Ser Gly Ile Val Glu Phe Trp Val Asp
100 105 110

Gly Lys Pro Arg Val Arg Lys Ser Leu Lys Lys Gly Tyr Thr Val Gly
115 120 125

Ala Glu Ala Ser Ile Ile Leu Gly Gln Glu Gln Asp Ser Phe Gly Gly
130 135 140

Asn Phe Glu Gly Ser Gln Ser Leu Val Gly Asp Ile Gly Asn Val Asn
145 150 155 160

Met Trp Asp Phe Val Leu Ser Pro Asp Glu Ile Asn Thr Ile Tyr Leu
165 170 175

Gly Gly Pro Phe Ser Pro Asn Val Leu Asn Trp Arg Ala Leu Lys Tyr
180 185 190

Glu Val Gln Gly Glu Val Phe Thr Lys Pro Gln Leu Trp Pro
195 200 205

<210> 2
<211> 16
<212> PRT
<213> Human

<400> 2
Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp Thr Ser
1 5 10 15

<210> 3
<211> 27
<212> PRT
<213> Human

<400> 3
Leu Val Gly Asp Ile Gly Asn Val Asn Met Trp Asp Phe Val Leu Ser
1 5 10 15

Pro Asp Glu Ile Asn Thr Ile Tyr Leu Gly Gly
20 25

<210> 4
<211> 12
<212> PRT
<213> Human

<400> 4
Leu Lys Lys Gly Tyr Thr Val Gly Ala Glu Ala Ser
1 5 10

<210> 5
<211> 10
<212> PRT
<213> Human

<400> 5
Arg Ala Leu Lys Tyr Glu Val Gln Gly Glu
1 5 10

<210> 6
<211> 486
<212> PRT
<213> Leuconostoc mesenteroides

<400> 6

Met Val Ser Glu Ile Lys Thr Leu Val Thr Phe Phe Gly Gly Thr Gly
1 5 10 15

Asp Leu Ala Lys Arg Lys Leu Tyr Pro Ser Val Phe Asn Leu Tyr Lys
20 25 30

Lys Gly Tyr Leu Gln Lys His Phe Ala Ile Val Gly Thr Ala Arg Gln
35 40 45

Ala Leu Asn Asp Asp Glu Phe Lys Gln Leu Val Arg Asp Ser Ile Lys
50 55 60

Asp Phe Thr Asp Asp Gln Ala Gln Ala Glu Ala Phe Ile Glu His Phe
65 70 75 80

Ser Tyr Arg Ala His Asp Val Thr Asp Ala Ala Ser Tyr Ala Val Leu
85 90 95

Lys Glu Ala Ile Glu Glu Ala Ala Asp Lys Phe Asp Ile Asp Gly Asn
100 105 110

Arg Ile Phe Tyr Met Ser Val Ala Pro Arg Phe Phe Gly Thr Ile Ala
115 120 125

Lys Tyr Leu Lys Ser Glu Gly Leu Leu Ala Asp Thr Gly Tyr Asn Arg
130 135 140

Leu Met Ile Glu Lys Pro Phe Gly Thr Ser Tyr Asp Thr Ala Ala Glu
145 150 155 160

Leu Gln Asn Asp Leu Glu Asn Ala Phe Asp Asp Asn Gln Leu Phe Arg
165 170 175

Ile Asp His Tyr Leu Gly Lys Glu Met Val Gln Asn Ile Ala Ala Leu
180 185 190

Arg Phe Gly Asn Pro Ile Phe Asp Ala Ala Trp Asn Lys Asp Tyr Ile
195 200 205

Lys Asn Val Gln Val Thr Leu Ser Glu Val Leu Gly Val Glu Glu Arg
210 215 220

Ala Gly Tyr Tyr Asp Thr Ala Gly Ala Leu Leu Asp Met Ile Gln Asn
225 230 235 240

His Thr Met Gln Ile Val Gly Trp Leu Ala Met Glu Lys Pro Glu Ser
245 250 255

Phe Thr Asp Lys Asp Ile Arg Ala Ala Lys Asn Ala Ala Phe Asn Ala
260 265 270

Leu Lys Ile Tyr Asp Glu Ala Glu Val Asn Lys Tyr Phe Val Arg Ala
275 280 285

Gln Tyr Gly Ala Gly Asp Ser Ala Asp Phe Lys Pro Tyr Leu Glu Glu
290 295 300

Leu Asp Val Pro Ala Asp Ser Lys Asn Asn Thr Phe Ile Ala Gly Glu
305 310 315 320

Leu Gln Phe Asp Leu Pro Arg Trp Glu Gly Val Pro Phe Tyr Val Arg
325 330 335

Ser Gly Lys Arg Leu Ala Ala Lys Gln Thr Arg Val Asp Ile Val Phe
340 345 350

Lys Ala Gly Thr Phe Asn Phe Gly Ser Glu Gln Glu Ala Gln Glu Ala
355 360 365

Val Leu Ser Ile Ile Ile Asp Pro Lys Gly Ala Ile Glu Leu Lys Leu
370 375 380

Asn Ala Lys Ser Val Glu Asp Ala Phe Asn Thr Arg Thr Ile Asp Leu
385 390 395 400

Gly Trp Thr Val Ser Asp Glu Asp Lys Lys Asn Thr Pro Glu Pro Tyr
405 410 415

Glu Arg Met Ile His Asp Thr Met Asn Gly Asp Gly Ser Asn Phe Ala
420 425 430

Asp Trp Asn Gly Val Ser Ile Ala Trp Lys Phe Val Asp Ala Ile Ser
435 440 445

Ala Val Tyr Thr Ala Asp Lys Ala Pro Leu Glu Thr Tyr Lys Ser Gly

450 455 460

Ser Met Gly Pro Glu Ala Ser Asp Lys Leu Leu Ala Ala Asn Gly Asp
465 470 475 480

Ala Trp Val Phe Lys Gly
485

<210> 7
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 7
ataaggggta caccatggtt tcagaaatca agacgttag 39

<210> 8
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 8

ttcccgggct ttaattaacc tttaaacacc 30

<210> 9

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 9

tggttggtta gctatggaaa aaccagaatc 30

<210> 10

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 10

taggatccag gtacgtctaa ttcttcaagg tatg 34

<210> 11

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 11

atggatccgc tgattctaaa aacaatacct tc 32

<210> 12

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 12

aagcttgcat gcctgcaggt tcccg 25

<210> 13

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 2, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 13

gatccgacat gtcgaggaag gcttttgtgt ttcccaaaga gtcggatact tccg 54

<210> 14

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 1 3

<400> 14

gatccggaag tatccgactc tttgggaaac acaaaagcct tcctcgacat gtcg 54

<210> 15

<211> 48

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for partial amino acids of Sequence 3, and a partial rest

riction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 15

gatccgtgct gtcaccagat gagattaaca ccatctatct tggcgggg 48

<210> 16

<211> 48

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 1 5

<400> 16

gatcccccgc caagatagat ggtgttaatc tcatctggtg acagcacg 48

<210> 17

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 4, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 17

gatccctgaa gaagggatac actgtggggg cagaagcaag cg 42

<210> 18

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 17

<400> 18

gatccgcttg cttctgcccc cacagtgtat cccttcttca gg 42

<210> 19

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 5, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 19

gatcccgggc actgaagtat gaagtgcaag gcgaag 36

<210> 20

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 1
9

<400> 20

gatccttcgc cttgcacttc atacttcagt gcccgg 36

<210> 21

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for partial amino acids of Sequence 3, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 21

gatcctagtg ggagacattg gaaatgtgaa catgtgggac tttgtgg 47

<210> 22

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 2
1

<400> 22

gatcccacaa agtcccacat gttcacattt ccaatgtctc ccactag 47

<210> 23

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 23

taggatccgt ctaattcttc aaggtatggc ttg 33

<210> 24

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 24

aaggatccgt acctgctgat tctaaaaaca atac 34

<210> 25

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<400> 25

ttggatccag caggtacgtc taattcttca ag 32

<210> 26

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 26

taggatccga ttctaaaaac aataccttca tcg 33

<210> 27

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 27

gggtgtttaa aggtggatcc taattaaagc ccgg 34

<210> 28

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 28

taggatcctt ctgaaccaaa gttaaacgtg cc 32

<210> 29

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide Primer

<400> 29

atggatccca agaagcacaa gaagctgtct tg 32

<210> 30

<211> 1024

<212> PRT

<213> Escherichia coli

<400> 30

Met Thr Met Ile Thr Asp Ser Leu Ala Val Val Leu Gln Arg Arg Asp
1 5 10 15

Trp Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala His Pro
20 25 30

Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro
35 40 45

Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg Phe Ala Trp Phe
50 55 60

Pro Ala Pro Glu Ala Val Pro Glu Ser Trp Leu Glu Cys Asp Leu Pro
65 70 75 80

Glu Ala Asp Thr Val Val Val Pro Ser Asn Trp Gln Met His Gly Tyr
85 90 95

Asp Ala Pro Ile Tyr Thr Asn Val Thr Tyr Pro Ile Thr Val Asn Pro
100 105 110

Pro Phe Val Pro Thr Glu Asn Pro Thr Gly Cys Tyr Ser Leu Thr Phe
115 120 125

Asn Val Asp Glu Ser Trp Leu Gln Glu Gly Gln Thr Arg Ile Ile Phe
130 135 140

Asp Gly Val Asn Ser Ala Phe His Leu Trp Cys Asn Gly Arg Trp Val
145 150 155 160

Gly Tyr Gly Gln Asp Ser Arg Leu Pro Ser Glu Phe Asp Leu Ser Ala
165 170 175

Phe Leu Arg Ala Gly Glu Asn Arg Leu Ala Val Met Val Leu Arg Trp
180 185 190

Ser Asp Gly Ser Tyr Leu Glu Asp Gln Asp Met Trp Arg Met Ser Gly
195 200 205

Ile Phe Arg Asp Val Ser Leu Leu His Lys Pro Thr Thr Gln Ile Ser
210 215 220

Asp Phe His Val Ala Thr Arg Phe Asn Asp Asp Phe Ser Arg Ala Val

225 230 235 240

Leu Glu Ala Glu Val Gln Met Cys Gly Glu Leu Arg Asp Tyr Leu Arg
245 250 255

Val Thr Val Ser Leu Trp Gln Gly Glu Thr Gln Val Ala Ser Gly Thr
260 265 270

Ala Pro Phe Gly Gly Glu Ile Ile Asp Glu Arg Gly Gly Tyr Ala Asp
275 280 285

Arg Val Thr Leu Arg Leu Asn Val Glu Asn Pro Lys Leu Trp Ser Ala
290 295 300

Glu Ile Pro Asn Leu Tyr Arg Ala Val Val Glu Leu His Thr Ala Asp
305 310 315 320

Gly Thr Leu Ile Glu Ala Glu Ala Cys Asp Val Gly Phe Arg Glu Val
325 330 335

Arg Ile Glu Asn Gly Leu Leu Leu Leu Asn Gly Lys Pro Leu Leu Ile
340 345 350

Arg Gly Val Asn Arg His Glu His His Pro Leu His Gly Gln Val Met
355 360 365

Asp Glu Gln Thr Met Val Gln Asp Ile Leu Leu Met Lys Gln Asn Asn
370 375 380

Phe Asn Ala Val Arg Cys Ser His Tyr Pro Asn His Pro Leu Trp Tyr
385 390 395 400

Thr Leu Cys Asp Arg Tyr Gly Leu Tyr Val Val Asp Glu Ala Asn Ile
405 410 415

Glu Thr His Gly Met Val Pro Met Asn Arg Leu Thr Asp Asp Pro Arg
420 425 430

Trp Leu Pro Ala Met Ser Glu Arg Val Thr Arg Met Val Gln Arg Asp
435 440 445

Arg Asn His Pro Ser Val Ile Ile Trp Ser Leu Gly Asn Glu Ser Gly
450 455 460

His Gly Ala Asn His Asp Ala Leu Tyr Arg Trp Ile Lys Ser Val Asp
465 470 475 480

Pro Ser Arg Pro Val Gln Tyr Glu Gly Gly Gly Ala Asp Thr Thr Ala
485 490 495

Thr Asp Ile Ile Cys Pro Met Tyr Ala Arg Val Asp Glu Asp Gln Pro
500 505 510

Phe Pro Ala Val Pro Lys Trp Ser Ile Lys Lys Trp Leu Ser Leu Pro
515 520 525

Gly Glu Thr Arg Pro Leu Ile Leu Cys Glu Tyr Ala His Ala Met Gly
530 535 540

Asn Ser Leu Gly Gly Phe Ala Lys Tyr Trp Gln Ala Phe Arg Gln Tyr
545 550 555 560

Pro Arg Leu Gln Gly Gly Phe Val Trp Asp Trp Val Asp Gln Ser Leu
565 570 575

Ile Lys Tyr Asp Glu Asn Gly Asn Pro Trp Ser Ala Tyr Gly Gly Asp
580 585 590

Phe Gly Asp Thr Pro Asn Asp Arg Gln Phe Cys Met Asn Gly Leu Val
595 600 605

Phe Ala Asp Arg Thr Pro His Pro Ala Leu Thr Glu Ala Lys His Gln
610 615 620

Gln Gln Phe Phe Gln Phe Arg Leu Ser Gly Gln Thr Ile Glu Val Thr
625 630 635 640

Ser Glu Tyr Leu Phe Arg His Ser Asp Asn Glu Leu Leu His Trp Met
645 650 655

Val Ala Leu Asp Gly Lys Pro Leu Ala Ser Gly Glu Val Pro Leu Asp
660 665 670

Val Ala Pro Gln Gly Lys Gln Leu Ile Glu Leu Pro Glu Leu Pro Gln
675 680 685

Pro Glu Ser Ala Gly Gln Leu Trp Leu Thr Val Arg Val Val Gln Pro

690 695 700

Asn Ala Thr Ala Trp Ser Glu Ala Gly His Ile Ser Ala Trp Gln Gln
705 710 715 720

Trp Arg Leu Ala Glu Asn Leu Ser Val Thr Leu Pro Ala Ala Ser His
725 730 735

Ala Ile Pro His Leu Thr Thr Ser Glu Met Asp Phe Cys Ile Glu Leu
740 745 750

Gly Asn Lys Arg Trp Gln Phe Asn Arg Gln Ser Gly Phe Leu Ser Gln
755 760 765

Met Trp Ile Gly Asp Lys Lys Gln Leu Leu Thr Pro Leu Arg Asp Gln
770 775 780

Phe Thr Arg Ala Pro Leu Asp Asn Asp Ile Gly Val Ser Glu Ala Thr
785 790 795 800

Arg Ile Asp Pro Asn Ala Trp Val Glu Arg Trp Lys Ala Ala Gly His
805 810 815

Tyr Gln Ala Glu Ala Ala Leu Leu Gln Cys Thr Ala Asp Thr Leu Ala
820 825 830

Asp Ala Val Leu Ile Thr Thr Ala His Ala Trp Gln His Gln Gly Lys
835 840 845

Thr Leu Phe Ile Ser Arg Lys Thr Tyr Arg Ile Asp Gly Ser Gly Gln
850 855 860

Met Ala Ile Thr Val Asp Val Glu Val Ala Ser Asp Thr Pro His Pro
865 870 875 880

Ala Arg Ile Gly Leu Asn Cys Gln Leu Ala Gln Val Ala Glu Arg Val
885 890 895

Asn Trp Leu Gly Leu Gly Pro Gln Glu Asn Tyr Pro Asp Arg Leu Thr
900 905 910

Ala Ala Cys Phe Asp Arg Trp Asp Leu Pro Leu Ser Asp Met Tyr Thr
915 920 925

Pro Tyr Val Phe Pro Ser Glu Asn Gly Leu Arg Cys Gly Thr Arg Glu
930 935 940

Leu Asn Tyr Gly Pro His Gln Trp Arg Gly Asp Phe Gln Phe Asn Ile
945 950 955 960

Ser Arg Tyr Ser Gln Gln Gln Leu Met Glu Thr Ser His Arg His Leu
965 970 975

Leu His Ala Glu Glu Gly Thr Trp Leu Asn Ile Asp Gly Phe His Met
980 985 990

Gly Ile Gly Gly Asp Asp Ser Trp Ser Pro Ser Val Ser Ala Glu Phe
995 1000 1005

Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln Leu Val Trp Cys Gln Lys
1010 1015 1020

<210> 31
<211> 448
<212> PRT
<213> Escherichia coli

<400> 31
Thr Pro Glu Met Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile
1 5 10 15

Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala Ala
20 25 30

Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile Leu Leu
35 40 45

Ile Gly Asp Gly Met Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr
50 55 60

Ala Glu Gly Ala Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu
65 70 75 80

Thr Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys Pro
85 90 95

Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala Thr Ala Trp Ser Thr Gly
100 105 110

Val Lys Thr Tyr Asn Gly Ala Leu Gly Val Asp Ile His Glu Lys Asp
115 120 125

His Pro Thr Ile Leu Glu Met Ala Lys Ala Ala Gly Leu Ala Thr Gly
130 135 140

Asn Val Ser Thr Ala Glu Leu Gln Asp Ala Thr Pro Ala Ala Leu Val
145 150 155 160

Ala His Val Thr Ser Arg Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu
165 170 175

Lys Cys Pro Gly Asn Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr
180 185 190

Glu Gln Leu Leu Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly Ala
195 200 205

Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly Glu Trp Gln Gly Lys Thr
210 215 220

Leu Arg Glu Gln Ala Gln Ala Arg Gly Tyr Gln Leu Val Ser Asp Ala
225 230 235 240

Ala Ser Leu Asn Ser Val Thr Glu Ala Asn Gln Gln Lys Pro Leu Leu
245 250 255

Gly Leu Phe Ala Asp Gly Asn Met Pro Val Arg Trp Leu Gly Pro Lys
260 265 270

Ala Thr Tyr His Gly Asn Ile Asp Lys Pro Ala Val Thr Cys Thr Pro
275 280 285

Asn Pro Gln Arg Asn Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp
290 295 300

Lys Ala Ile Glu Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu Gln
305 310 315 320

Val Glu Gly Ala Ser Ile Asp Lys Gln Asp His Ala Ala Asn Pro Cys
325 330 335

Gly Gln Ile Gly Glu Thr Val Asp Leu Asp Glu Ala Val Gln Arg Ala
340 345 350

Leu Glu Phe Ala Lys Lys Glu Gly Asn Thr Leu Val Ile Val Thr Ala
355 360 365

Asp His Ala His Ala Ser Gln Ile Val Ala Pro Asp Thr Lys Ala Pro
370 375 380

Gly Leu Thr Gln Ala Leu Asn Thr Lys Asp Gly Ala Val Met Val Met

385 390 395 400

Ser Tyr Gly Asn Ser Glu Glu Asp Ser Gln Glu His Thr Gly Gln Leu
405 410 415

Arg Ile Ala Ala Tyr Gly Pro His Ala Ala Asn Val Val Gly Leu Thr
420 425 430

Asp Gln Thr Asp Leu Phe Tyr Thr Met Lys Ala Ala Leu Gly Leu Lys
435 440 445

<210> 32
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for partial amino acids of Sequence 2,and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 32
gatccgacat gtcgaggaag gcttttgtgt ttcccaaaga gtcgg 45

<210> 33
<211> 45
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 3
2

<400> 33

gatcccgact ctttgggaaa cacaaaagcc ttcctcgaca tgtcg 45

<210> 34

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of
the DNA coding for partial amino acids of Sequence 2,and a partial rest
riction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 34

gatccaggaa ggcttttgtg tttcccaaag agtcgg 36

<210> 35

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 3
4

<400> 35

gatcccgact ctttgggaaa cacaaaagcc ttcctg 36

<210> 36

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 36

cacaggaaac agaccatggg atccgtttca gaaatc 36

<210> 37

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 37

ttggatccat caccggcacc atattgtgca cg 32

<210> 38

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 38

aaggatcctc agctgacttc aagccatacc ttg 33

<210> 39

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 39

aaggatccaa ggtatggctt gaagtcagct g 31

<210> 40

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 40

aaggatccaa ggtatggctt gaagtcagct g 31

<210> 41

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 41

ggtacgtata attcatcaag gtatggcttg 30

<210> 42

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 42

tatacgtacc tgctgattct aaaaac 26

<210> 43

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for partial amino acids of Sequence 2 .

<400> 43

aggaaggctt ttgtgtttcc caaagagtcg 30

<210> 44

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 43

<400> 44

cgactctttg ggaaacacaa aagccttcct 30

<210> 45

<211> 55

<212> PRT

<213> Hepatitis B virus

<400> 45

Met Gln Trp Asn Ser Thr Ala Phe His Gln Ala Leu Gln Asp Pro Arg

1 5 10 15

Val Arg Gly Leu Tyr Phe Pro Ala Gly Gly Ser Ser Ser Gly Thr Val

20 25 30

Asn Pro Ala Pro Asn Ile Ala Ser His Ile Ser Ser Ile Ser Ala Arg

35 40 45

Thr Gly Asp Pro Val Thr Asn

50 55

<210> 46

<211> 12

<212> PRT

<213> Hepatitis B virus

<400> 46

Asp Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly

1 5 10

<210> 47

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 46, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 47

gatccgaccc gcgtgttcgt ggtctgtatt tcccggctgg tg 42

<210> 48

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 47

<400> 48

gatccaccag ccgggaaata cagaccacga acacgcgggt cc 42

<210> 49

<211> 84

<212> PRT

<213> Human

<400> 49

Ala Val Ser Glu Ile Gln Phe Met His Asn Leu Gly Lys His Leu Ser

1 5 10 15

Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His

20 25 30

Asn Phe Val Ala Leu Gly Ala Ser Ile Ala Tyr Arg Asp Gly Ser Ser

35 40 45

Gln Arg Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His Gln

50 55 60

Lys Ser Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Ile Lys

65 70 75 80

Ala Lys Pro Gln

<210> 50

<211> 15

<212> PRT

<213> Human

<400> 50

Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn

1 5 10 15

<210> 51

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 50, and a partial restriction site of BamHI consisting of 5' end of "gatcc" and 3' end of "g".

<400> 51

gatccgaacg tgttgaatgg ctgcgtaaaa aactgcagga cgttcataac g 51

<210> 52

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 51

<400> 52

gatccgttat gaacgtcctg cagtttttta cgcagccatt caacacgttc g 51

<210> 53

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 53

tatgaccatg attacggatt cactggcc 28

<210> 54

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 54

ctgcccggtt attattattt ttgacaccag 26

<210> 55

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 55

taggatccta cgccaatgtc gttatccagc g 31

<210> 56

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 56

ttggatccag tgaagcgacc cgcattgacc 30

【００３１】

【図面の簡単な説明】

【図１】本発明のハイブリッド酵素Ｇ３０８Ｃ１の抗ＣＲＰ抗体測定能を示すグラフである。。

【図２】本発明のハイブリッド酵素Ｇ３０８Ｃ１のＣＲＰ測定能を示すグラフである。なお、図２から１４において

回復率(%)={(各CRP濃度時のG6PDH活性)-(CRP 0mg/dl時のG6PDH活性)}/{(抗体非存在時のG6PDH活性)-(CRP 0mg/dl時のG6PDH活性)}X100

活性比(%)={抗体存在時のG6PDH活性 / 抗体非存在時のG6PDH活性} X100 である。

【図３】本発明のハイブリッド酵素Ｇ３０８Ｃ２のＣＲＰ測定能を示すグラフである。

【図４】本発明のハイブリッド酵素Ｇ３０８Ｃ３のＣＲＰ測定能を示すグラフである。

【図５】本発明のハイブリッド酵素Ｇ３０８Ｃ５のＣＲＰ測定能を示すグラフである。

【図６】本発明のハイブリッド酵素Ｇ３０６Ｃ１のＣＲＰ測定能を示すグラフである。

【図７】本発明のハイブリッド酵素Ｇ３０９Ｃ１のＣＲＰ測定能を示すグラフである。

【図８】本発明のハイブリッド酵素Ｇ３６２Ｃ１の抗ＣＲＰ抗体測定能を示すグラフである。

【図９】本発明のハイブリッド酵素Ｇ３０６Ｃ１、Ｇ３０６Ｃ１５、Ｇ３０６Ｃ１８のＣＲＰ測定感度を比較したグラフである。

【図10】本発明のハイブリッド酵素Ｇ３０６ｄ３Ｃ１のＣＲＰ測定能を示すグラフである。

【図11】本発明のハイブリッド酵素Ｇ３０６Ｈ１の抗ｐｒｅＳ２抗体測定能を示すグラフである。

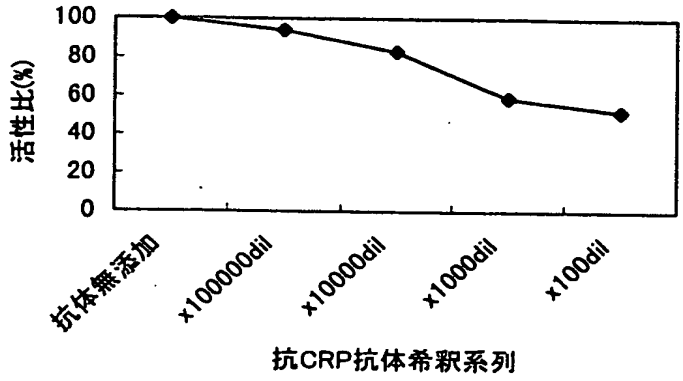
【図12】本発明のハイブリッド酵素Ｇ３０６Ｐ１のＰＴＨ測定能を示すグラフである。

【図13】本発明のハイブリッド酵素Ｂ７９６Ｃ１の抗ＣＲＰ抗体測定能を示すグラフである。

【図14】本発明のハイブリッド酵素Ｂ７９６Ｃ１のＣＲＰ測定能を示すグラフである。

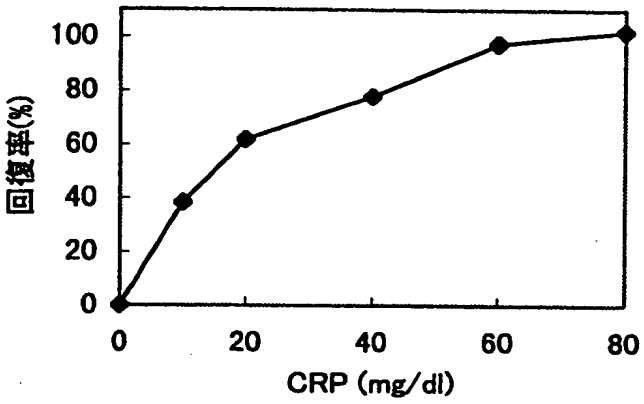
【書類名】　図面

【図１】

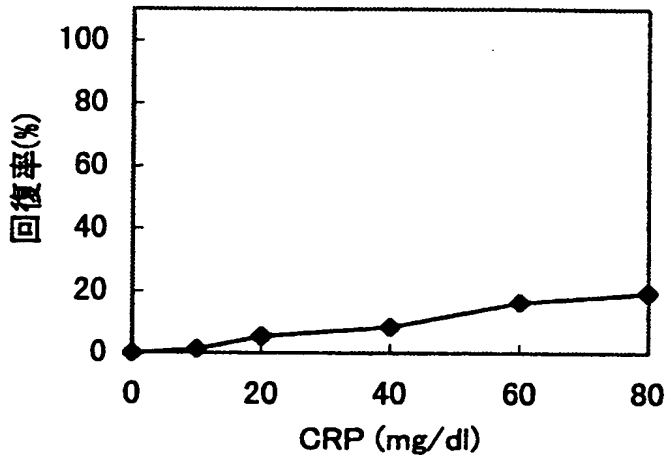


活性比（%）＝ (抗体存在時のG6PDH活性 / 抗体非存在時のG6PDH活性) × 100

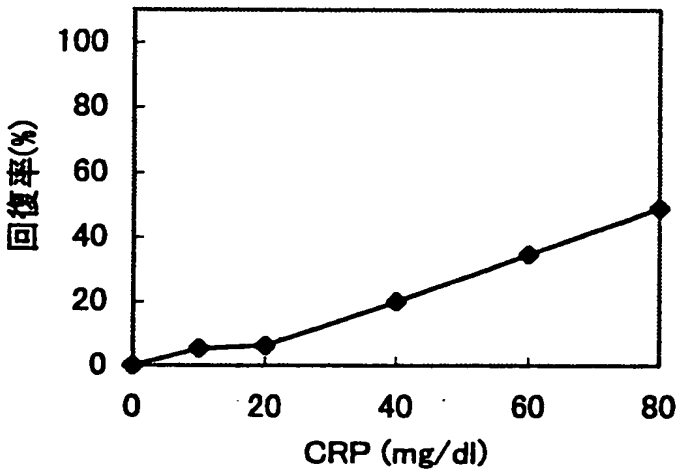
【図２】



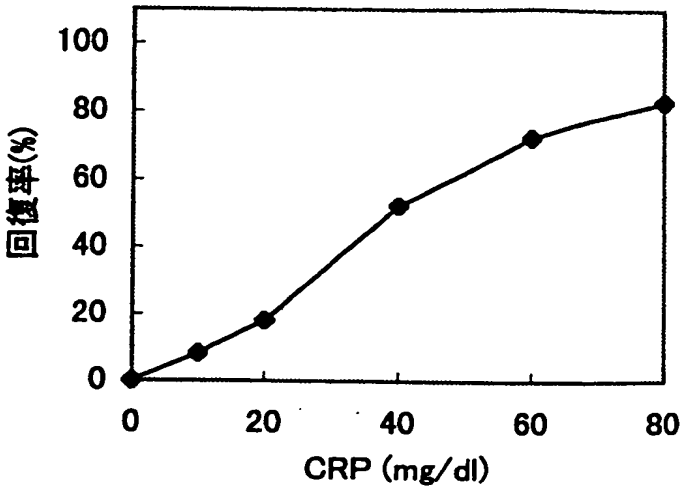
【図３】


100
80
60
40
20
0
回復率(%)
0
20
40
60
80
CRP (mg/dl)

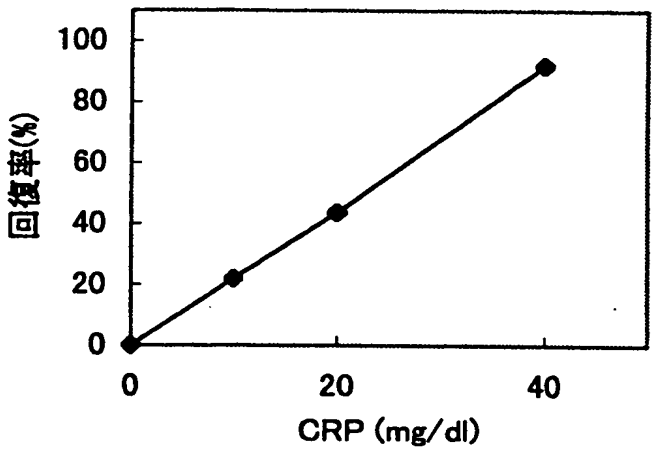
【図４】


100
80
60
40
20
0
回復率(%)
0
20
40
60
80
CRP (mg/dl)

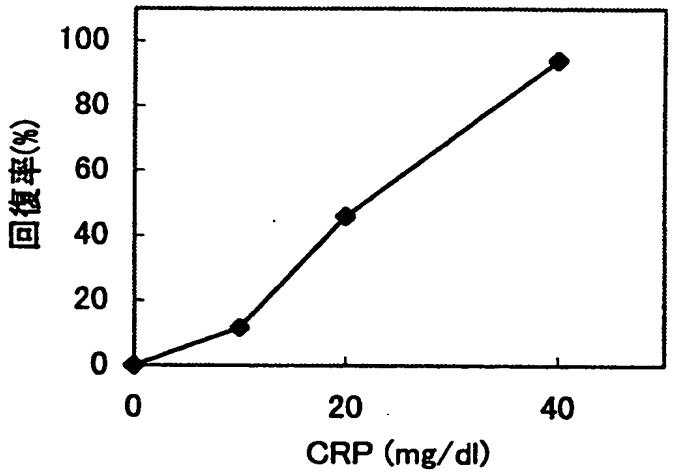
【図５】


100
80
60
40
20
0
回復率(%)
0
20
40
60
80
CRP (mg/dl)

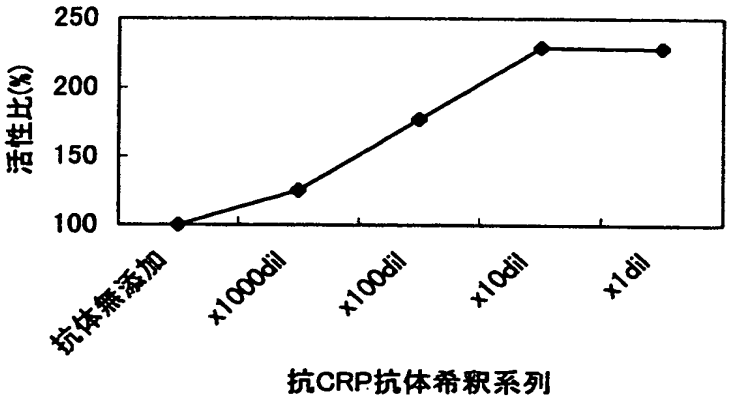
【図６】


100
80
60
40
20
0
回復率(%)
0
20
40
CRP (mg/dl)

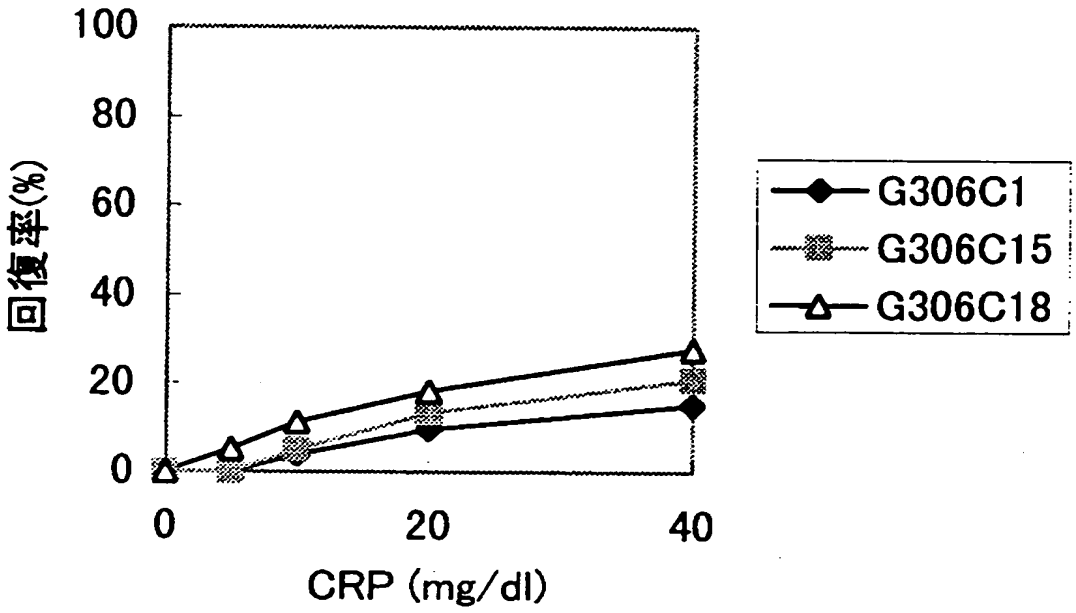
【図７】


100
80
60
40
20
0
回復率(%)
0
20
40
CRP (mg/dl)

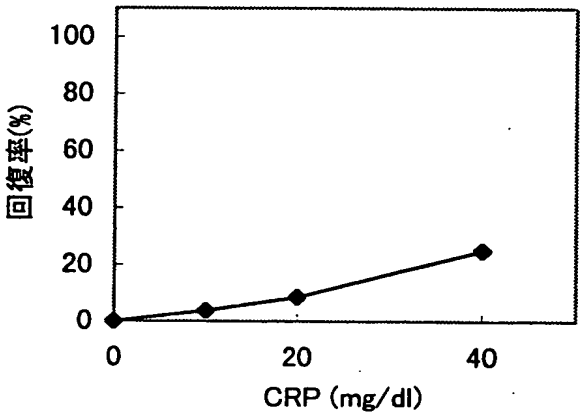
【図８】


250
200
150
100
活性比(%)
抗体無添加
x1000dil
x100dil
x10dil
x1dil
抗CRP抗体希釈系列

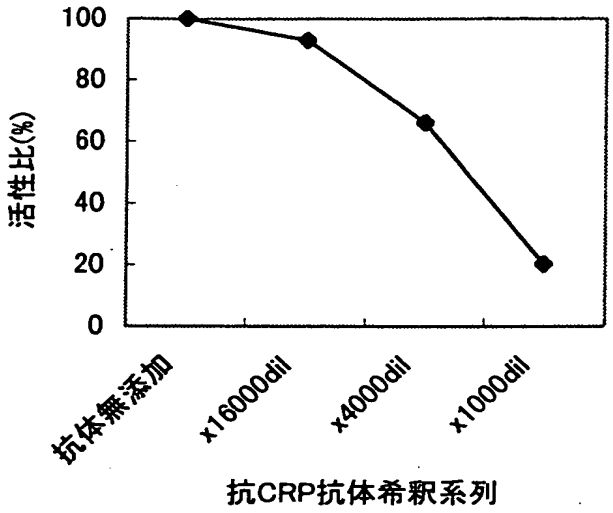
【図９】


100
80
60
40
20
0
回復率(%)
0
20
40
CRP (mg/dl)
G306C1
G306C15
G306C18

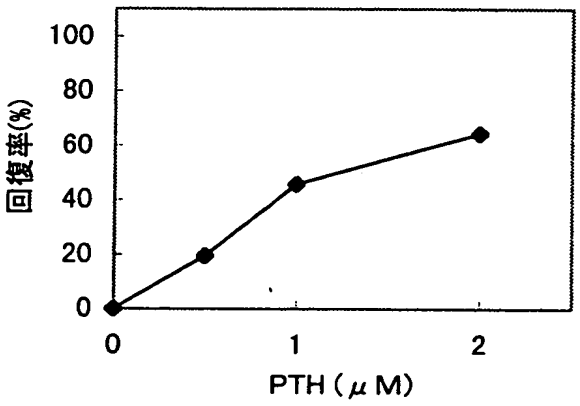
【図１０】


100
80
60
40
20
0
回復率(%)
0
20
40
CRP (mg/dl)

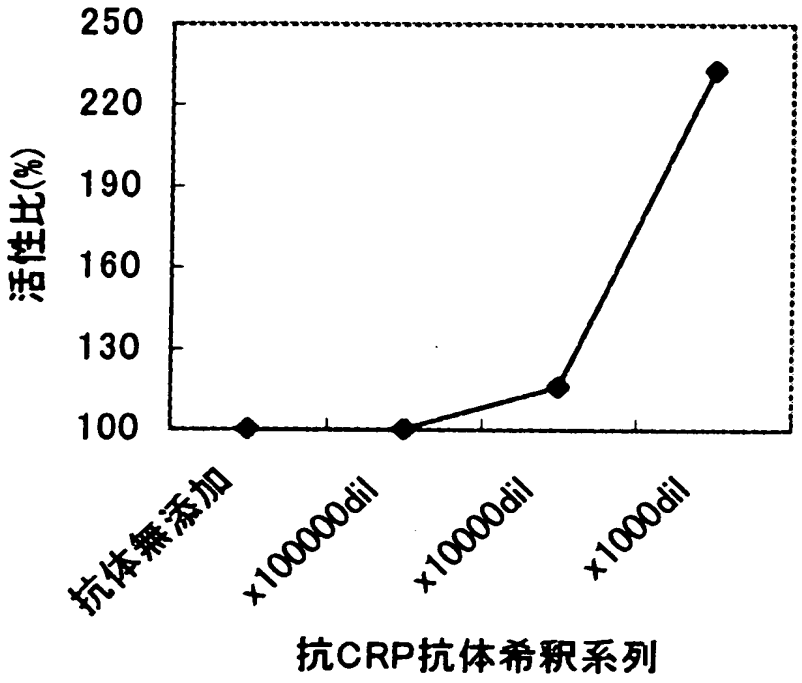
【図１１】


100
80
60
40
20
0
活性比(%)
抗体無添加
x16000dil
x4000dil
x1000dil
抗CRP抗体希釈系列

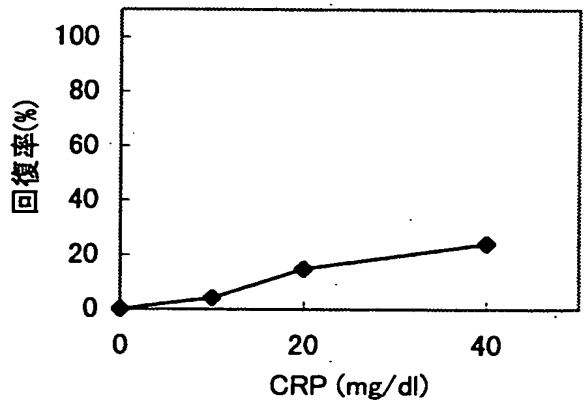
【図１２】


100
80
60
40
20
0
回復率(%)
0
1
2
PTH (μM)

【図１３】


250
220
190
160
130
100
活性比(%)
抗体無添加
x100000dil
x10000dil
x1000dil
抗CRP抗体希釈系列

【図１４】


100
80
60
40
20
0
回復率(%)
0
20
40
CRP (mg/dl)

【書類名】　要約書

【要約】

【課題】　ホモジニアスな比色法で試料中の微量なＣＲＰ測定を可能とする方法を提供すること、および高分子物質をホモジニアスな系で測定する方法を提供することである。

【解決手段】　G6PDHに於ける、外来ペプチドが挿入されても同様の酵素活性を有し、且つ外来ペプチド部分に結合能を有する物質が結合した場合、当該酵素活性が変化し得る、特定位置を見出し、当該位置にＣＲＰ由来のペプチドを挿入しハイブリッド酵素を提供し、このものと試料とを接触させたときの酵素活性の変化をみることにより、ホモジニアスな比色法で試料中の微量なＣＲＰ測定を可能とした。

【選択図】なし

認定・付加情報

特許出願の番号	特願２０００－２７４２１９
受付番号	５００００１１５４５５６
書類名	特許願
担当官	仲村　百合子　　１７３０
作成日	平成１２年１０月２３日

＜認定情報・付加情報＞

【提出日】	平成12年 9月 8日
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【代理人】	申請人
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【氏名又は名称】	大多和　明敏
【代理人】	
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次頁無

出証特２００１－３０３８７８０

出　願　人　履　歴　情　報

識別番号　　〔０００２５２３００〕

１．変更年月日　　１９９０年　８月　７日
［変更理由］　新規登録
住　所　　大阪府大阪市中央区道修町３丁目１番２号
氏　名　　和光純薬工業株式会社

JAPAN PATENT OFFICE

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application : September 8, 2000
Application Number : No.274219/2000
Applicant :Wako Pure Chemical Industries, Ltd.

May 11,2001

Commissioner,
Japan Patent Office Kozo Oikawa

Certificate No. 2001-3038780

【NAME OF DOCUMENT】 Patent request

【REFERENCE NUMBER】 WJ018

【ADDRESSEE】 To the Commissioner of the JPO

【INTERNATIONAL PATENT CLASSIFICATION】

C12N 9/00

C12N 15/52

G01N 33/50

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【NAME】 Akitoshi Otawa

【AGENT】

【IDENTIFICATION NUMBER】 100086999

【PATENT ATTORNEY】

【NAME】 Akiko Otawa

【DECLARATION OF PRIORITY】

【APPLICATION NUMBER】 No.174604/2000

【FILING DATE】 June 12, 2000

【INDICATION OF FEE】

【DEPOSIT ACCOUNT NUMBER】 014742

【FEE】 21,000yen

【LIST OF ANNEXED DOCUMENT】

【DOCUMENT】 Specification 1

【DOCUMENT】 Drawing 1

【DOCUMENT】 Abstract 1

【DOCUMENT】 Letter of Attorney 1

【MATTER MENTIONED SPECIALLY OF THE DOCUMENTS FOR SUBMIT】

The Letter of Attorney is submited by Supplement.

【REQUIREMENT OF PROOF】 Request

【Title of Document】 specification

【Title of the Invention】 HYBRID ENZYME AND USE THEREOF

【Scope of Claim for a Patent】

【Claim 1】 A hybrid enzyme which has a partial substitution or an insertion of a peptide containing a part of an amino acid sequence represented by SEQ ID NO:1, in which said hybrid enzyme has the same enzyme activity as an original enzyme without the substitution or the insertion of said peptide, and said hybrid enzyme activity is modulated when a material having binding ability to said peptide introduced by the substitution or the insertion is bound to the peptide moiety.

【Claim 2】 The hybrid enzyme according to claim 1, in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from the amino acid sequence of SEQ ID NO: 1.

【Claim 3】 The hybrid enzyme according to claim 2, in which the peptide has a property of being capable of binding to a material having binding ability to C-reactive protein.

【Claim 4】 The hybrid enzyme according to claim 1, in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from any one of SEQ ID NO: 2 through SEQ ID NO: 5.

【Claim 5】 The hybrid enzyme according to claim 1, in which the original enzyme is a glucose-6-phosphate dehydrogenase, a β-galactosidase or an alkaline phosphatase.

【Claim 6】 The hybrid enzyme according to claim 1, in which the material having binding ability to the peptide is an antibody.

【Claim 7】 A reagent for measurement of C-reactive protein comprising the hybrid enzyme according to any one of claims 1 through 6.

【Claim 8】 The reagent according to claim 7 further comprising an anti-C-reactive protein antibody.

【Claim 9】 A kit for measurement of C-reactive protein containing a reagent

comprising the hybrid enzyme according to any one of claims 1 through 6.

【Claim 10】 The kit according to claim 9 further comprising an anti-C-reactive protein antibody.

【Claim 11】 A method for measurement of C-reactive protein which is characterized in using the hybrid enzyme according to any one of claims 1 through 6.

【Claim 12】 The method according to claim 11 further comprising using an anti-C-reactive protein antibody in combination.

【Claim 13】 A method for measurement of C-reactive protein comprising bringing a sample containing C-reactive protein, the enzyme according to any one of claims 1 through 6 and an anti-C-reactive protein antibody into contact with one another, then measuring activity of the enzyme, and determining the amount of C-reactive protein in the sample based on the resulting enzyme activity.

【Claim 14】 A glucose-6-phosphate dehydrogenase, into which a peptide is introduced at a specific position by insertion or substitution.

【Claim 15】 The hybrid enzyme according to claim 14, in which the specific position is a position at which the glucose-6-phosphate dehydrogenase activity can be maintained even by the insertion or substitution of a peptide having 6 or more amino acid residues.

【Claim 16】 The hybrid enzyme according to claim 14, in which the specific position is a position at which the glucose-6-phosphate dehydrogenase activity is modulated when a material having binding ability to the peptide introduced by insertion or substitution is bound to said peptide.

【Claim 17】 The hybrid enzyme according to claim 14, in which the specific position is any position selected from the group consisting of the Asp294 position, the Leu302 to Asp310 positions, the Glu362 position, the N-terminal and the C-terminal of the amino acid sequence of glucose-6-phosphate dehydrogenase represented by SEQ ID NO: 6.

【Claim 18】 The hybrid enzyme according to claim 14, in which the peptide is

selected from the amino acid sequence of C-reactive protein.

【Claim 19】 The hybrid enzyme according to claim 14, in which the peptide has a character that there is a material having binding ability specifically to the peptide.

【Claim 20】 A reagent for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

【Claim 21】 A kit for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

【Claim 22】 A method for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which is characterized in using the hybrid enzyme according to any one of claims 14 through 19.

【Claim 23】 A method for measurement of a material containing the peptide, which comprises using the hybrid enzyme according to any one of claims 14 through 19 in combination with a material having binding ability to the peptide introduced into the hybrid enzyme by insertion or substitution.

【Claim 24】 A method for measurement of a material containing said peptide introduced into the hybrid enzyme according to any one of claim 14 through 19, which comprises bringing the hybrid enzyme according to any one of claims 14 through 19, a sample containing a material containing the peptide introduced into said hybrid enzyme by insertion or substitution and a material having binding ability to said peptide into contact with one another, then measuring activity of said hybrid enzyme, and determining the amount of the material containing said peptide in the sample based on the resulting enzyme activity.

【Claim 25】 A reagent for measurement of a material having binding ability to the peptide introduced into said hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

【Claim 26】 A kit for measurement of a material having binding ability to the peptide introduced into said hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises the hybrid enzyme according to any one of claims 14 through 19.

【Claim 27】 A method for measurement of a material having binding ability to the peptide introduced into said hybrid enzyme according to any one of claim 14 through 19 by insertion or substitution, which comprises using the hybrid enzyme according to any one of claims 14 through 19.

【Claim 28】 A method for measurement of a material having binding ability to said peptide introduced into the hybrid enzyme according to any one of claim 14 through 19, which comprises bringing the hybrid enzyme according to any one of claims 14 through 19 into contact with a sample containing a material having binding ability to the peptide, then measuring an activity of said hybrid enzyme, and determining the amount of the material having binding ability to said peptide in the sample based on the resulting enzyme activity.

【Claim 29】 A gene coding for a hybrid enzyme comprising an amino acid sequence into which a foreign peptide is introduced by substitution or insertion at any position selected from the group consisting of the Asp294 position, the Leu302 to Asp310 positions, the Glu362 position, the N-terminal and the C-terminal of the amino acid sequence of glucose-6-phosphate dehydrogenase represented by SEQ ID NO: 6.

【Claim 30】 A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 29 into a vector DNA.

【Claim 31】 A transformant or a transductant comprising the recombinant DNA

according to claim 30.

【Claim 32】 A method for producing a protein having enzyme activity of glucose-6-phosphate dehydrogenase and a property that the glucose-6-phosphate dehydrogenase activity is modulated when a material having binding ability to an amino acid sequence introduced into glucose-6-phosphate dehydrogenase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant according to claim 31, and collecting the protein.

【Claim 33】 A gene coding for a hybrid enzyme comprising an amino acid sequence into which an amino acid sequence, which can be cleaved with a restriction enzyme, is introduced by substitution or insertion at any position selected from the group consisting of the Asp294 position, the Leu302 to Asp310 positions, the Glu362 position, the N-terminal and the C-terminal of the amino acid sequence of glucose-6-phosphate dehydrogenase represented by SEQ ID NO: 6.

【Claim 34】 A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 33 into a vector DNA.

【Claim 35】 A β-galactosidase into which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced at a specific position by insertion or substitution.

【Claim 36】 The hybrid enzyme according to claim 35, in which the specific position is either selected from the Ile280 to Asp281 positions and Val796 to Ser797 positions of an amino acid sequence of a β-galactosidase represented by SEQ ID NO: 30.

【Claim 37】 A gene coding for the hybrid enzyme according to claim 36.

【Claim 38】 A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 37 into a vector DNA.

【Claim 39】 A transformant or a transductant comprising the recombinant DNA according to claim 38.

【Claim 40】 A method for producing a protein having an enzyme activity of a β-

galactosidase and a property that the β-galactosidase activity is modulated when a material having binding ability to an amino acid sequence introduced into the β-galactosidase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant according to claim 39, and collecting the protein.

【Claim 41】 An alkaline phosphatase into which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced at a specific position by insertion or substitution.

【Claim 42】 The hybrid enzyme according to claim 41, in which the specific position is any one selected from the Lys167 to Cys168 positions, the Cys168 to Tyr169 positions, the Glu407 to Asp408 positions, the Lys91 to Thr93 positions and the Tyr169 to Lys177 positions of an amino acid sequence of an alkaline phosphatase represented by SEQ ID NO: 31.

【Claim 43】 A gene coding for the hybrid enzyme according to claim 42.

【Claim 44】 A recombinant DNA, which is characterized in inserting the hybrid enzyme gene according to claim 43 into a vector DNA.

【Claim 45】 A transformant or a transductant comprising the recombinant DNA according to claim 44.

【Claim 46】 A method for producing a protein having an enzyme activity of an alkaline phosphatase and a property that the alkaline phosphatase activity is modulated when a material having binding ability to an amino acid sequence introduced into the alkaline phosphatase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant according to claim 45, and collecting the protein.

【Detailed Description of the Invention】

【0001】

【Technical Field of the Invention】

The present invention relates to a hybrid enzyme and a biological substance assay system using the same. More particularly, the present invention relates to a hybrid enzyme containing a part of an amino acid sequence of C-reactive protein (hereinafter briefly referred to as CRP) and CRP assay system using the hybrid enzyme, and a hybrid type of glucose-6-phosphate dehydrogenase (G6PDH) containing an foreign peptide at a specific position and a various foreign peptide assay system using the hybrid type enzyme.

【0002】

【Prior Art】

Biological substance assay systems utilizing the antigen-antibody reaction can be roughly divided into two types: homogeneous assay systems such as turbidimetric immunoassay and latex turbidimetric immunoassay, and heterogeneous assay systems such as an immunoassay using HPLC, electrophoresis etc., radio immunoassay and enzyme immunoassay (EIA). In either case, measurements are made at present using almost automated instruments. However, in the heterogeneous assay systems such as a macromolecule material assays or a small molecule material assays, special instruments are indispensable because solid phases are used. Accordingly, general-purpose automatic analyzers can not be applied to these systems. Also in the homogeneous assay systems, except for turbidimetric immunoassay and latex turbidimetric immunoassay, special instruments are used, and general-purpose automatic analyzers can not be applied to these systems. On the other hand, the EMIT method (IMMUNOASSAYS, R. M. Nakamura et al., Alan R. Liss, Inc., New York) using an enzyme is established and comes in practice at present as a homogeneous immunoassay system applicable to the general-purpose automatic analyzers. This assay system is a method for measurement of the amount of a material to be assayed by using an enzyme chemically modified with the material to be assayed and utilizing that enzyme activity is inhibited to result in a reduction in enzyme activity when an antibody to the material to

be assayed is bound thereto. However, this system has the problem that the material to be assayed is limited to a small molecule material such as haptene. Then, recently, a homogeneous assay system using a hybrid enzyme formed by inserting a foreign peptide into an enzyme by genic recombination is contrived as a homogeneous immunoassay system which makes it possible to assay a macromolecule material by use of the enzyme. That is to say, this is a method for assaying the amount of the foreign peptide, a material containing the peptide in its amino acid sequence, or a material having binding ability to the peptide, by utilizing that enzyme activity varies when an antibody is bound to a foreign peptide moiety in the hybrid enzyme. As the enzymes used in this method, there are known alkaline phosphatases (Published Japanese Translation of PCT International Publication (Toku-Hyo-Hei) 8-507686 (1996)) and β -galactosidases (FEBS Letters, 434, 23-27 (1998), FEBS Letters, 438, 267-271 (1998)). In this method, however, it is also known that the hybrid enzyme sometimes loses enzyme activity according to the position where a foreign peptide is inserted, or that even when the hybrid enzyme has enzyme activity, the enzyme activity sometimes does not vary when the material having binding ability is bound to the hybrid enzyme.

Meanwhile, CRP, which is β -globulin in the serum which reacts with C polysaccharide of Streptococcus Pneumoniae, is known to be acute phase reactant protein and to increase in an inflammation or tissue injury. The measurement of CRP is useful for early diagnosis of inflammation in tissue and diagnosis of diseases. Conventionally, CRP has been measured by turbidimetric immunoassay, latex turbidimetric immunoassay, enzyme immunoassay or radio immunoassay. Of these, turbidimetric immunoassay and latex turbidimetric immunoassay are applicable to the general-purpose automatic analyzers. However, as both are a method for detecting changes in turbidity, they are not said to be a complete homogeneous assay systems and have a problem with regard to accuracy on measurement. Accordingly, it has been desired to develop the micromeasurement of CRP applicable to the general-purpose

automatic analyzers according to the complete homogeneous assay system with high in assay accuracy.

【0003】

【Problems to be Solved by the Invention】

In view of the situation as described above, one of problems to be solved by the present invention is to provide a method which makes it possible to assay a trace amount of CRP in a sample by a homogeneous colorimetry. It is another problem to be solved by the present invention to provide a method for measuring a macromolecule material in a homogeneous system.

【0004】

【Means for Solving the Problems】

As a result of intensive research for solving the above-mentioned problems, the present inventors have found that the use of the above-mentioned hybrid enzyme makes it possible to assay in a homogeneous system a macromolecule material to be assayed, and have considered to be capable of developing a new CRP micromeasuring method applicable to the general-purpose automatic analyzers by application of this method to the assay of CRP, because the detection thereof can be conducted by a colorimetry. The present inventors have conducted further research, and have discovered that the above-mentioned problems can be solved by using a hybrid enzyme containing a part of a material to be analyzed such as CRP at a certain position of some kind of enzyme, thus completing the present invention.

That is to say, as an effective hybrid enzyme for providing a method which makes it possible to measure a trace amount of CRP in a sample by a homogeneous colorimetry using a hybrid enzyme in which a CRP-derived peptide is inserted into a specific position, the present invention provides (1) a hybrid enzyme which has a partial substitution or an insertion of a peptide containing a part of an amino acid sequence represented by SEQ ID NO:1, in which said hybrid enzyme has the same enzyme

activity as an original enzyme without the substitution or the insertion of said peptide, and said hybrid enzyme activity varied when a material having binding ability to said peptide introduced by the substitution or the insertion is bound to the peptide moiety; (2) the hybrid enzyme described in (1), in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from the amino acid sequence of SEQ ID NO: 1; (3) the hybrid enzyme described in (2), in which the peptide has a property of being capable of binding to a material having binding ability to CRP; (4) the hybrid enzyme described in (1), in which the peptide comprises an amino acid sequence having at least 6 or more sequential amino acid residues selected from any one of SEQ ID NO: 2 through SEQ ID NO: 5; (5) the hybrid enzyme described in (1), in which the enzyme is a glucose-6-phosphate dehydrogenase (hereinafter sometimes referred to as G6PDH), a β-galactosidase or an alkaline phosphatase; and (6) the hybrid enzyme described in (1), in which the material having binding ability to the peptide is an antibody. As a method for measuring a trace amount of CRP in a sample by a homogeneous colorimetry using the above-mentioned hybrid enzyme, the present invention provides (7) a reagent for measurement of CPR comprising the enzyme described in any one of (1) through (6); (8) the reagent described in (7) further comprising an anti-CRP antibody; (9) a kit for measurement of CRP containing a reagent comprising the enzyme described in any one of (1) through (6); (10) the kit described in (9) further comprising an anti-CRP antibody; (11) a method for measurement of CRP which is characterized in using the enzyme described in any one of (1) through (6); (12) the method described in (11) further comprising using an anti-CRP antibody in combination; and (13) a method for measurement of CRP comprising bringing a sample containing CRP, the enzyme described in any one of (1) through (6) and an anti-CRP antibody into contact with one another, then determining an activity of the enzyme, and determining the amount of CRP in the sample based on the resulting enzyme activity.

Further, as G6PDH-containing hybrid enzyme having a similar enzyme activity also when a foreign peptide is inserted therein, and having a property that the enzyme activity can vary when a material having binding ability to the foreign peptide moiety is bound thereto, for measurement of a macromolecule material in a homogeneous system, the present invention provides (14) G6PDH having a peptide introduced into a specific position of G6PDH by insertion or substitution; (15) the enzyme described in (14), in which the specific position is a position at which the G6PDH activity can be maintained even in the insertion or substitution of a peptide having 6 or more amino acid residues; (16) the hybrid enzyme described in (14), in which the specific position is a position at which the G6PDH activity is modulated when a material having binding ability to the peptide introduced by insertion or substitution is bound to the peptide; (17) the enzyme described in (14), in which the specific position is any position selected from the group consisting of the Asp294, the Leu302 to Asp310, Glu362, the N-terminal and the C-terminal of the amino acid sequence of G6PDH represented by SEQ ID NO: 6; (18) the enzyme described in (14), in which the peptide is selected from the amino acid sequence of CRP; and (19) the enzyme described in (14), in which the peptide has a character that there is a material having binding ability specifically to the part of the hybrid enzyme in which the peptide is substituted or inserted. The present invention further provides (20) a reagent comprising the enzyme described in any one of (14) through (19), for measurement of a material containing the peptide introduced into the hybrid enzyme described in any one of (14) through (19) by insertion or substitution; (21) a kit comprising the enzyme described in any one of (14) through (19), for measurement of a material containing the peptide introduced into the hybrid enzyme according to any one of (14) through (19) by insertion or substitution; (22) a method comprising using the hybrid enzyme described in any one of (14) through (19), for measurement of a material containing the peptide introduced into the enzyme according to any one of (14) through (19) by insertion or substitution; (23) a method comprising using the enzyme described

in any one of (14) through (19) in combination with a material having binding ability to the peptide introduced into the enzyme described in any one of (14) through (19) by insertion or substitution, for measurement of a material containing the peptide; (24) a method comprising bringing the enzyme described in any one of (14) through (19), a sample containing a material containing the peptide introduced into the enzyme by insertion or substitution and a material having binding ability to the peptide into contact with one another, then measuring activity of the enzyme, and determining the amount of the material containing the peptide in the sample based on the resulting enzyme activity, for measurement of the material containing the peptide; (25) a reagent comprising the enzyme described in any one of (14) through (19), for measurement of a material having binding ability to the peptide introduced into the enzyme by insertion or substitution; (26) a kit comprising the enzyme described in any one of (14) through (19), for measurement of a material having binding ability to the peptide introduced into the enzyme by insertion or substitution; (27) a method comprising using the enzyme described in any one of (14) through (19), for measurement of a material having binding ability to the peptide introduced into the enzyme by insertion or substitution; and (28) a method comprising bringing the enzyme described in any one of (14) through (19) into contact with a sample containing a material having binding ability to the peptide introduced into the enzyme by insertion or substitution, then measuring an activity of the enzyme, and determining the amount of the material having binding ability to said peptide in the sample based on the resulting enzyme activity, for measurement of a material having binding ability to the peptide.

In the present invention, in order to produce a reagent for detecting a certain material or a material having binding ability thereto, it has been discovered that (29) a gene coding for a hybrid enzyme comprising an amino acid sequence into which a foreign peptide is introduced by substitution or insertion at any position selected from the group consisting of the Asp294, the Leu302 to Asp310, Glu362, the N-terminal and the C-

terminal of the amino acid sequence of G6PDH represented by SEQ ID NO: 6 is useful. The present invention further provides (30) a novel recombinant DNA which is characterized in inserting the hybrid enzyme gene described in (29) into a vector DNA; (31) a transformant or a transductant comprising the recombinant DNA described in (30); (32) a method for producing a protein having an enzyme activity of G6PDH and a property that the G6PDH activity is modulated when a material having binding ability to an amino acid sequence introduced into G6PDH by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant described in (31), and collecting the protein; (33) a gene coding for a hybrid enzyme comprising an amino acid sequence into which an amino acid sequence ,which can be cleaved with a restriction enzyme is introduced by substitution or insertion at any position selected from the group consisting of the Asp294 position, the Leu302 through Asp310 positions, the Glu362 position, the N-terminal and the C-terminal of the amino acid sequence of G6PDH represented by SEQ ID NO: 6; and (34) a novel recombinant DNA which is characterized in inserting the hybrid enzyme gene described in (33) into a vector DNA.

Still further, the present invention provides (35) β-galactosidase in which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced into a specific position of β-galactosidase by insertion or substitution; (36) the β-galactosidase described in (35), in which the specific position is a site selected from the position Ile280/Asp281 and Val796/Ser797 of an amino acid sequence of a β-galactosidase represented by SEQ ID NO: 30; (37) a gene coding for the hybrid enzyme described in (36); (38) a novel recombinant DNA which is characterized in inserting the hybrid enzyme gene described in (37) into a vector DNA; (39) a transformant or a transductant comprising the recombinant DNA described in (38); and (40) a method for producing a protein having an enzyme activity of a β-galactosidase and a property that the β-galactosidase activity varies when a material having binding

ability to an amino acid sequence introduced into the β-galactosidase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant described in (39), and collecting the protein.

Furthermore, the present invention provides (41) alkaline phosphatase in which a peptide selected from an amino acid sequence represented by SEQ ID NO: 1 is introduced into a specific position of an alkaline phosphatase by insertion or substitution; (42) the hybrid enzyme described in (41), in which the specific position is a site selected from the position Lys167/168Cys, Cys168/169Tyr, Glu407/408Asp, Lys91/93Thr and Tyr169/177Lys of an amino acid sequence of an alkaline phosphatase represented by SEQ ID NO: 31; (43) a gene coding for the hybrid enzyme described in (42); (44) a novel recombinant DNA , which is characterized in inserting the hybrid enzyme gene described in (43) into a vector DNA; (45) a transformant or a transductant comprising the recombinant DNA described in (44); and (46) a method for producing a protein having an enzyme activity of an alkaline phosphatase and having a property that the alkaline phosphatase activity varies when a material having binding ability to an amino acid sequence introduced into the alkaline phosphatase by substitution or insertion is bound to the amino acid sequence, which comprises cultivating the transformant or the transductant described in (45), and collecting the protein.

【0005】

【The Embodiments of the Invention】

Origin enzymes used for the hybrid enzymes of the present invention may be any, as long as they are enzymes generally used. Examples thereof include adenosine deaminases, alkaline phosphatases, α-amylases, bacterial luciferases, β-galactosidases, β-galactosidase fragments, β-lactamases, carbonic anhydrases, catalases, firefly luciferases, glucose oxidases, glucose-6-phosphate dehydrogenases, glucosidases, hexokinases, horseradish peroxidases, invertases, isocitrate dehydrogenases, lysozymes, malate dehydrogenases, micro peroxidases, 6-phosphofructases and xanthine oxidases.

Further, all enzymes can be used, as long as each amino acid sequence or DNA sequence arrangement thereof is determinate or can be determined, and the enzyme activity varies when a part or plural parts of the enzyme are substituted by a foreign peptide or a foreign peptide is inserted into a part or plural parts thereof and a material having binding ability to the peptide is bound to the peptide. Among which, enzymes having high enzyme activity, ones having good stability and ones which can be assayed by the colorimetry are particularly preferred. Examples thereof include G6PDHs, β-galactosidases and alkaline phosphatases. Genes of these enzymes are available by cloning methods from genomes usually employed. Of course, already cloned genes or synthetic DNA can also be used. These enzymes may be derived from any, and each amino acid sequence thereof may be a sequence obtained by deletion, substitution or addition of one or more amino acids, as long as they have intrinsic enzyme activity.

The foreign peptide used for preparing the hybrid enzyme of the present invention may be any, as long as a material such as an antibody or a receptor having binding ability to the peptide is present. Donors thereof include, for example, biological substances such as CRP, IgG, IgA, IgM, C3, C4, β2 microglobulin and albumin, various cancer markers such as α-fetoprotein, CA19-9, prostatic specific antigen (PSA) and carcinoembryonic antigen (CEA), various hormones such as insulin, human chorionic gonadotropin (hCG), albumin, streptolysin O (SLO), prolactin, parathyroid hormone and thyroid stimulating hormone (TSH), various toxins such as streptolysin O (SLO), or various viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) and human papilloma virus (HPV), which are generally assayed(measured) in immunoassay systems.

There is no particular limitation on the vector used in the recombinant DNA of the present invention, as long as it is a vector such as a plasmid vector and a bacterio phage vector, which can be replicated and inherited in various hosts of procaryotic cells and/or eucaryotic cells. Examples of such vectors include Escherichia coli-derived

plasmids such as pBR322, pBR325, pUC12, pUC13 and pBluescript, yeast-derived plasmids such as pSH19 and pSH15, and Bacillus subtilis-derived plasmids such as pUB110, pTP5 and pC194, which are generally available in this field. Further, examples of the phage vectors include bacteriophages such as λ phage, and viruses of animals and insects such as retroviruses, vaccinia viruses and nuclear polyhedrosis viruses.

The host cells used for the production of the hybrid enzymes of the present invention include bacteria (for example, Escherichia coli), yeast (for example, Saccharomyces), animal cells (for example, chinese hamster cell CHO) and insect cells (for example, BmN4).

Construction of recombinant DNA, and expression and purification of the hybrid enzyme for producing the hybrid enzymes of the present invention may be carried out by known methods, for example, methods described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory).

There is no particular limitation on an expression vector, as long as it can be replicated and inherited in various hosts of procaryotic cells and/or eucaryotic cells, and has the function of expressing genes the hybrid enzymes from cloned gene in various hosts of procaryotic cells and/or eucaryotic cells, that is to say, the function of producing the desired hybrid enzymes. For example, the vectors generally available in this field are preferably pBR322, pUC12, pUC13, pTrcHis, pTrc99A, pMAL-c2 and artificially modified products thereof (DNA fragments obtained by treating the vectors with appropriate restriction enzymes) when the host cell is ESCHERICHIA coli; pRS403, pRS404, pRS413, pRS414 and pYES2 when the host cell is yeast; plasmids pRSVneo ATCC37224, pSV2dhfr ATCC37145, pdBPV-MMTneo ATCC37224 and pSV2neo ATCC37149 when the host cell is an animal cell; and Autographica california nuclear polyhedrosis virus (AcNPV) and Bombyx mori nuclear polyhedrosis virus (BmNPV) when the host cell is an insect cell.

In the following, the preparation of the hybrid enzyme of the present invention is described as to the case that G6PDH is used as the original enzyme, a CRP-derived peptide as the foreign peptide, and Escherichia coli as the host cell.

The G6PDH gene can be obtained, for example, by the following method. That is to say, first, cells are harvested from a culture product of Leuconostoc mesenteroides by centrifugation according to a conventional method described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory), and genomic DNA is extracted. Operations are hereinafter conducted according to well-known techniques generally employed, which are described in Molecular Cloning(J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory) and the like, unless otherwise specified. Using the above-mentioned genomic DNA as a template, an oligonucleotide primer having an N-terminal sequence and a C-terminal sequence of the G6PDH gene sequence shown in R.Levy et al., J. Biological Chemistry, 266, 13028-(1991), or a sequence upstream therefrom and a sequence downstream therefrom is added, and the polymerase strand reaction is conducted by using a DNA thermal cycler (Perkin Elmer) to specifically amplify a DNA fragment containing the G6PDH gene. The resulting DNA fragment is integrated into a vector DNA according to a conventional method, thereby obtaining recombinant DNA containing DNA coding for G6PDH.

Further, for connecting the foreign peptide to the G6PDH gene by insertion or substitution, any method may be used. For example, the method for preparing a deletion mutant using an exonuclease described in "Laboratory Manual Genetic Engineering" (edited by Seikan Muramatsu, the 3rd edition, pages 219 to 230, Maruzen), the method for introducing artificial mutation such as the Kunkel method, the cassette method or the method using the PCR, or the phosphorothioate method described in "DNA Cloning 1" (edited by D.M. Glover et al., 2nd Edition, pages 197 to 228, Takara), the gap double stranded DNA method and the MHT protocol can be used in

combination with each other. The resulting DNA coding for the hybrid enzyme is normally inserted into the vector DNA according to conventional method, thereby being able to obtain the recombinant DNA containing the DNA coding for the hybrid enzyme. Then, a method for preparing a restriction enzyme site in the G6PDH gene inserted into the plasmid, and connecting the foreign peptide by insertion or substitution is described below.

That is to say, using the G6PDH gene as a template, primers to which a site cleavable with a restriction enzyme such as BamH I (BamH I site) is added in order to insert the BamH I site into the 5'-terminal of base sequences coding for amino acid sequences on both sides of a insertion position of a foreign peptide in G6PDH, is subjected to the PCR, in combination with primers of base sequences coding for upstream part or downstream part from a position where a foreign peptide is to be inserted respectively, to amplify DNA fragments to which the BamH I site is inserted. Then, two kinds of fragments thus obtained are connected to each other on the vector, thereby being able to construct the G6PDH gene into which the BamH I site is inserted. The restriction enzyme site is cleaved with the restriction enzyme, thereby being able to insert any DNA fragment having a complementary sequence and the cleaved site at both ends thereof.

When any amino acid sequence is eliminated for inserting the peptide by substitution, both sides of the sequence to be eliminated are subjected to the same operation as described above. Thus, the G6PDH gene connected to the foreign peptide by substitution can be obtained.

When the recombinant DNA is constructed, secretory production can also be conducted as a fused protein of the hybrid enzyme and another protein or peptide. Further, the fused protein produced by secretion can also be cleaved with an appropriate protease or by chemical treatment to obtain the hybrid enzyme. Examples of the proteins to be fused include maltose binding proteins and glutathione S-transferase, and

examples of the peptides to be fused include histidine tags and FLAG tags.

The basic vectors for the construction of the recombinant DNA include, for example, plasmid vectors such as pBR322 (J. G. Sutcliffe, Cold Spring Harbor Symposium, 43, 77 (1979)), pUC18/19 (C. Yanisch-Perron et al., Gene, 33, 102-119 (1985)), pBluescript IISK+ (STRATEGENE), pMAL-C2 (NEW England Biolabs), pTrc99A (Amersham Pharmacia), pKK223-3 (Amersham Pharmacia) and pET-11 (STRATEGENE), and bacteriophage vectors such as λENBL3 (STRATEGENE) and λ DASHII (Funakoshi).

A promoter used in the recombinant DNA may be any, as long as it functions in Escheria. coli. Examples thereof include a lac promoter, a trp promoter, a T7 promoter and derivatives thereof. Further, the recombinant DNA may contain an initiation signal such as a liposome binding sequence functioning in E. coli and a terminator. It may further contain a selective marker gene such as an ampicillin-resistance gene or a tetracycline-resistant gene.

【0006】

Using the recombinant DNA thus constructed, E. coli is transformed or transduced to prepare a transformant or a transductant. E. coli includes M103, JA221, HB101, C600, XL1-Blue and JM109.

Methods for transforming or transducing the recombinant DNA into Escherichia coli include, for example, the method of Cohen et al. (Proc. Natl. Acad. Sci. U.S.A., 69, 2110 (1972)) and the method of Hanahan et al. (J. Mol. Biol., 166, 557 (1983)). The recombinant DNA may be obtained from the recombinant DNA-containing transformant or transductant by a conventional method such as the alkali miniprep method.

The hybrid enzyme of the present invention can be produced by cultivating the transformant or transductant of the recombinant DNA prepared as described above. Media used include, for example, Luria-Bertani medium (Molecular Cloning (J.

Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory)), 2×YT medium (Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory)) and M9 medium (J. Miller, Exp. Mol. Genet., Cold Spring Harbor Laboratory, New York, page 431 (1972)). The pH of the medium is preferably from 5 to 8. The cultivation is conducted usually at 14 to 42°C, preferably at 28 to 39°C, for 3 to 24 hours, optionally with aeration or stirring. Further, an expression inducing reagent such as isopropyl-β-D-1-thiogalacto pyranoside, or an antibiotic such as ampicillin or chloramphenicol may be added as needed.

The hybrid enzyme of the present invention can be obtained in the following manner from the culture product obtained by the above-mentioned cultivation. That is to say, when the hybrid enzyme exists in a culture solution of the culture product, a culture filtrate or culture supernatant containing the hybrid enzyme is obtained by a conventional method such as filtration or centrifugation. On the other hand, when the hybrid enzyme exists in periplasms or cells of the cultivated transformant or transductant, the culture product is subjected to a conventional method such as filtration or centrifugation to collect the periplasms or cells, which is suspended in a proper buffer solution and the cells in this solution are disrupted by a conventional method such as ultrasonication, lysozyme treatment or freeze-thawing. Then, a crude extract solution containing the hybrid enzyme is obtained by a conventional method such as filtration or centrifugation.

The hybrid enzyme may be separated and purified from the thus obtained culture filtrate, culture supernatant or crude extract solution containing the hybrid enzyme of the present invention by a suitable combination of known separating and purifying methods. These known separating and purifying methods include methods mainly utilizing a difference in solubility such as salting-out and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods

utilizing a difference in electric charge such as ion exchange chromatography, methods utilizing a difference in hydrophobicity such as hydrophobic chromatography, methods utilizing a difference in isoelectric point such as isoelectric point electrophoresis, and methods utilizing specific affinity such as affinity chromatography.

The effect of the anti-CRP antibody to the enzyme activity of the resulting hybrid enzyme can be examined, for example, in the following manner. That is to say, the enzyme activity of the hybrid enzyme is assayed in the absence and presence of the anti-CRP antibody, respectively, to examine modulations in activity due to the binding of the anti-CRP antibody. To 6 μl of a solution obtained by diluting each hybrid enzyme solution with 100 mM Tris/HCl buffer (pH 7.8) containing 1% bovine albumin, 3 mM magnesium chloride and 150 mM sodium chloride to about 1 U/ml, 150 μl of 100 mM Tris/HCl buffer containing 3 mM magnesium chloride and 150 mM sodium chloride (pH 7.8, hereinafter referred to as buffer A for brevity), or an antibody solution obtained by diluting 100 times anti-CPR goat antibody with buffer A is added, followed by reaction at 37°C for 5 minutes. Then, 75 μl of buffer A containing 10 mM glucose-6-phosphate (G6P) and 6 mM nicotinamide adenine dinucleotide (NAD) are added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes are determined as G6PDH activity.

【0007】

The foreign peptide inserted into the enzyme may be any, as long as it maintains the structure that a material having binding ability to the peptide can bind thereto, and its enzyme activity is maintained even by insertion of the peptide. However, as described in Antibodies A Laboratory Manual (Ed Harlow et al., pages 76-, Cold Spring Harbor Laboratory), the peptide to be inserted is required to have at least 6 or more sequential amino acid residues for maintaining its antigenicity. When the foreign peptide to be inserted is a CRP-derived peptide, all or a part of the whole amino acid sequence of CRP represented by SEQ ID NO: 1 is used. Of these, an amino acid

sequence containing 6 to 50 sequential amino acid residues, for example, a sequential amino acid sequence selected from Gln(1)-Asp(16), Glu(14)-Ala(24), Leu(22)-Ser(45), Thr(41)-Asn(61), Arg(47)-Ile(63), Lys(114)-Lys(121), Glu(130)-Glu(138), Ile(134)-Gly(148), Gln(137)-Leu(152), Glu(147)-Leu(152), Asp(3)-Ser(18), Leu(152)-Val(165), Val(165)-Gly(178), Leu(121)-Ser(132) and Arg(188)-Glu(197), is preferred. In particular, an amino acid sequence containing at least 6 or more sequential amino acid residues selected from sequences represented by SEQ ID NO: 2 (Asp(3)-Ser(18)), SEQ ID NO: 3 (Leu(152)-Gly(178)), SEQ ID NO: 4 (Leu(121)-Ser(132)) and SEQ ID NO: 5 (Arg(188)-Glu(197)) is more preferred. The peptide moiety introduced into the enzyme by insertion or substitution may contain a sequence other than the desired peptide, such as a restriction enzyme site, which is sometimes introduced in the course of preparing the hybrid enzyme.

In the present invention, the foreign peptide may be inserted into the origin enzyme of the hybrid enzyme at any position, as long as the enzyme activity is maintained also when the foreign peptide is inserted, and is modified when a material having binding ability to the inserted foreign peptide is bound to the peptide. The positions considered to be suitable therefor include a site exposed on a surface of the enzyme and a site where activation is influenced. The preferred position at which the foreign peptide is inserted into G6PDH as the origin enzyme was studied using the CRP-derived peptide. That is to say, we tried to insert the foreign peptide into the N-terminal, 32/33 (which means "between the 32nd and the 33rd", hereinafter the same), 37/38, 48/49, 66/67, 87/88, 139/140, 226/227, 294/295, 296/297, 302/303, 305-310, 362/363, 409/410 and the C-terminal of G6PDH. Results thereof show that the effective sites for CRP measurement at which the enzyme activity remains in the insertion of the peptide and is modulated by the anti-CRP antibody reaction are the N-terminal, 139/140, 302/303, 305/306, 306/307, 308/309, 309/310, 362/363 and the C-terminal, as shown in Table 1.

【0008】

【Table 1】

Insertion Site	Activity in Peptide Insertion	Modulations in Enzyme Activity in Antibody Reaction
Lys32/Lys33	-	-
Gln37/Lys38	-	-
Gln48/Ala49	+	-
Phe66/Thr67	+	-
Val87/Thr88	-	-
Gly226/Tyr227	-	-
Ala296/Asp297	-	-
Leu305/Asp306	+	+
Asp306/Val307	+	+
Pro308/Ala309	+	+
Ala309/Asp310	+	+
Glu329/Gly330	-	-
Glu362/Gln363	+	+
Lys409/Lys410	-	-
C-terminal	+	+

The partial substitution of the amino acid sequence of the enzyme by the foreign peptide means that amino acid residues at specific sites of the enzyme are substituted by the amino acid sequence of the foreign peptide. In this case, the amino acid residue of the peptide introduced by substitution may be either more or less than the amino acid residues eliminated from the enzyme, as long as the enzyme activity of the original enzyme is maintained also after substitution, and is modulated when a material having binding ability to the foreign peptide introduced by substitution is

bound to the peptide. It is preferred that the number of the amino acid residues eliminated is approximately the same as that of the amino acid residues introduced. Further, it is preferred that the number of the amino acid residues of the peptide introduced by substitution is approximately the same as that of the amino acid residues of the foreign peptide introduced into the above-mentioned enzyme. Still further, the origin enzyme may be substituted by the foreign peptide at any position, as long as the enzyme activity is maintained also after substitution, and is modulated when a material having binding ability to the foreign peptide introduced by substitution is bound to the peptide. The position at which the foreign peptide is introduced by substitution is selected, based on the position at which the foreign peptide can be inserted into the original enzyme of the hybrid enzyme, as shown above.

【0009】

In the present invention, the hybrid enzyme obtained as described above is used for qualitative analysis or quantitative analysis

The hybrid enzyme of the present invention is modulated in its enzyme activity according to the binding amount thereof, when a material having binding ability to the foreign peptide introduced by substitution or insertion is bound to the peptide. Accordingly, the presence and amount of the material having the binding ability to the foreign peptide can be detected by allowing a sample containing the binding material to react with the hybrid enzyme of the present invention, and assaying modulations in the enzyme activity. The use of the hybrid enzyme of the present invention and the material having the binding ability allows the presence or amount of the material containing the peptide to be detected, by utilizing that the amount of the material having the binding ability which is bound to the hybrid enzyme is modulated by competition of the peptide introduced into the hybrid enzyme by insertion or substitution and the substance containing the peptide to the material having the binding ability. This process comprises the steps of (1) bringing a sample containing a material to be

analyzed, the hybrid enzyme of the present invention and the substance having the binding ability to the foreign peptide introduced into the hybrid enzyme of the present invention by insertion or substitution in contact with one another to form a reaction mixture, (2) bringing the reaction mixture into contact with a substrate to the starting enzyme, and (3) monitoring changes in the enzyme activity of the hybrid enzyme according to the amount of the material to be analyzed existing in the reaction mixture. Step (2) can also be carried out after the reaction mixture is allowed to reach a steady state or an equilibrium state, and step (1) can be carried out sequentially or concurrently. In step (1), a sample containing the material having the binding ability to the peptide can be reacted with the hybrid enzyme, thereby detecting the presence and amount of the material having the binding ability to the peptide.

As described above, the use of the hybrid enzyme-containing reagent of the present invention allows the presence or amount of the anti-CRP antibody to be directly assayed. Further, the use of a reagent containing the anti-CRP antibody together with the hybrid enzyme of the present invention makes it possible to conduct the assay for indirectly detecting the presence or amount of CRP as an antigen, by binding competition of the hybrid enzyme and CRP to the anti-CRP antibody as a binding material.

The enzyme activity of the hybrid enzyme may be assayed in accordance with the method for assaying the activity of the original enzyme.

The G6PDHs used for preparing G6PDH-containing hybrid enzymes for assaying a macromolecule material in a homogeneous system, which can maintain the same enzyme activity also after insertion of the foreign peptide and be modulated in the enzyme activity when the material having binding ability to the foreign peptide moiety is bound to the foreign peptide moiety, include one having the amino acid sequence represented by SEQ ID NO: 6 or a sequence obtained by deletion, substitution or addition of one or more amino acids of the amino acid sequence, and one having

G6PDH activity even though it is different origin. Preferred examples of the positions at which the foreign peptides are inserted include the positions described above.

The foreign peptides include all the peptides described above. Of these, the peptides as described above are preferably used as CRP.

The hybrid enzyme prepared by using an enzyme other than the G6PDH, into which the foreign peptide containing as a part thereof a CRP-derived peptide is introduced by insertion or substitution, is also prepared in the same manner as described above. The position at which the foreign peptide is introduced by insertion or substitution may also be appropriately selected in the same manner. When the β-galactosidase is used as the enzyme, the position may also be selected, based on the descriptions of FEBS Letters, 434, 23-27 (1998) and FEBS Letters, 438, 267-271 (1998). When the alkaline phosphatase is used, the position may also be selected, based on a method described in Proc. Natl. Acad. Sci. U.S.A., 92 (1995).

The use of the thus obtained hybrid enzyme into which the foreign peptide is introduced allows the presence or the amount of the material having the binding ability to the foreign peptide to be directly assayed, and the use of the hybrid enzyme and the material having the binding ability to the foreign peptide such as an antibody in combination makes it possible to conduct the assay for indirectly detecting the presence or the amount of the macromolecule material containing the foreign peptide. These assays may be conducted, based on the assaying operations of CRP or the anti-CRP antibody described above. Further, the use of the hybrid enzyme allows the macromolecule material to be assayed with high sensitivity in the homogeneous system, and this method can also be applied to general-purpose automatic analyzers.

Examples are shown below for illustrating the invention in more detail, but the invention is not construed as being limited by descriptions given therein.

【0010】

【Example】

EXAMPLE 1

Construction Plasmid containing G6PDH gene

Five milliliters of LACTOBACILLI MRS BROTH (DIFICO) was inoculated with Leuconostoc mesenteroides, and shake cultured at 26°C for 16 hours to obtain a culture medium. Then, the culture medium was centrifuged at 4°C at 6000 rpm for 10 minutes, and harvested to obtain cells. The cells were suspended in a 10 mM tris (hydroxymethyl)aminomethane (Tris/HCl) buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) (hereinafter referred to as TE for brevity), and achromopeptitase was added so as to give a final concentration of 300 u/ml, followed by standing at 37°C for 2 hours. Then, SDS and Proteinase K were added so as to give final concentrations of 0.5% and 100 μg/ml, respectively, and the resulting suspension was further allowed to stand at 37°C for 2 hours to conduct bacteriolysis. Genomic DNA of Leuconostoc mesenteroides was extracted as a donor of a G6PDH gene according to a conventional method described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory). Operations were hereinafter conducted according to well-known techniques generally employed, which were described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory) and the like, unless otherwise specified. Then, for obtaining the G6PDH gene, the polymerase strand reaction (hereinafter referred to as PCR for brevity) was conducted according to the following procedure. The above-mentioned genomic DNA (10 ng) was added as a template DNA, and 0. 1 nmol of each oligonucleotide primer represented by SEQ ID NO: 7 and SEQ ID NO: 8, respectively, containing the N-terminal and C-terminal sequences of the G6PDH gene, respectively, was added. Then, the reaction cycle at 94°C for 30 seconds, at 65°C for 30 seconds and at 72°C for 4 minutes was repeated 25 times using a DNA thermal cycler (Perkin Elmer) to amplify DNA fragment. As a result, an about 1.5-kbp DNA fragment containing the G6PDH gene was specifically amplified. The resulting DNA fragment was ligated to an Eco

RV site of cloning vector pBluescript II KS+ (Stratagene) to construct plasmid pBSWG.

Then, cloning vector pUC18 was digested with Eco RI and Sal I, and then, an end thereof was made flush. The resulting DNA fragment was ligated to the end made flush to construct plasmid pUCG which could express the G6PDH gene. Further, the 750th from the N-terminal of the G6PDH gene, cytosine, was varied to thymine using the oligonucleotide primer represented by SEQ ID NO: 9 and Mutan-K (Takara Shuzo Co., Ltd.) according to the Kunkel method, thereby constructing plasmid pBSMG containing the G6PDH gene having no restriction enzyme Nco I recognition sequence at a position other than the N-terminal, without changing the amino acid sequence. This plasmid was digested with restriction enzymes Nco I and Pst I, and an about 1.5-kbp G6PDH gene was recovered. This gene was ligated to an about 2.7-kbp DNA fragment obtained by digesting plasmid pUCG with restriction enzymes Nco I and Pst I to construct plasmid pUCMG.

【0011】

EXAMPLE 2

Construction of Recombinant DNA Coding for Fused Enzyme Having Human CRP-Derived Peptide Ligated between Pro308/Ala309 of G6PDH

Using plasmid pUCMG of Example 1 as a template, and using the oligonucleotide primer represented by SEQ ID NO: 7, and the oligonucleotide primer represented by SEQ ID NO: 10, in which a restriction enzyme Bam H I recognition sequence was added to the 5'-end side of an anti-sense strand sequence upstream from Pro308, the PCR was conducted to obtain an about 0.9-kbp DNA fragment containing a part from the N-terminal to Pro308 of the G6PDH gene, in which the restriction enzyme Bam H I recognition sequence was ligated to a downstream site. Similarly, using the oligonucleotide primer represented by SEQ ID NO: 11, in which a restriction enzyme Bam HI recognition sequence was added to the 5'-end side of an anti-sense chain sequence downstream from Ala309, and the oligonucleotide primer represented by SEQ

ID NO: 12 containing the C-terminal anti-sense strand sequence of the G6PDH gene, an about 0.6-kbp DNA fragment containing a part from Ala309 to the C-terminal of the G6PDH gene, in which the restriction enzyme BamH I recognition sequence was ligated to an upstream site was obtained.

The fragment of the N-terminal side was digested with restriction enzymes BamH I and Nco I, and the fragment of the C-terminal side was digested with restriction enzymes BamH I and Pst I, followed by ligation with an about 2.7-kbp DNA fragment obtained by digesting plasmid pUCMG with restriction enzyme Nco I and Pst I. Thus, recombinant pUCMG308B having a BamH I site sequence only at Pro308/Ala309 of the G6PDH gene was constructed. This recombinant was cleaved with restriction enzyme BamH I, and synthetic polynucleotides (a combination of SEQ ID NO: 13 and SEQ ID NO: 14, complemental with each other) having DNA coding for the amino acid sequence represented by SEQ ID NO: 2 were ligated thereto to construct pUCMG308C1. Synthetic nucleotides (a combination of SEQ ID NO: 15 and SEQ ID NO: 16, and a combination of SEQ ID NO: 21 and SEQ ID NO: 22, complemental with each other) having DNA coding for a part (portion) of the amino acid sequence represented by SEQ ID NO: 3 were each ligated to construct pUCMG308C2 and pUCMG308C13. Further, synthetic nucleotides (a combination of SEQ ID NO: 17 and SEQ ID NO: 18, complemental with each other) having DNA coding for the amino acid sequence represented by SEQ ID NO: 4 were ligated to construct pUCMG308C3, and synthetic nucleotides (a combination of SEQ ID NO: 19 and SEQ ID NO: 20, complemental with each other) having DNA coding for the amino acid sequence represented by SEQ ID NO: 5 were ligated to construct pUCMG308C5. The respective synthetic nucleotides have the following complemental structures with each other:

【Chemical Formula 1】

5'-gatccgacatgtcgaggaaggcttttgtgtttcccaaagagtcggatacttccg -3' SEQ ID NO: 13

3'- gatccggaagtatccgactctttgggaaacacaaaagccttcctcgacatgtcg-5' SEQ ID NO: 14

5'-gatccgtgctgtcaccagatgagattaacaccatctatcttggcgggg -3' SEQ ID NO: 15

3'- gatcccccgccaagatagatggtgttaatctcatctggtgacagcacg-5' SEQ ID NO: 16

5'-gatccctgaagaagggatacactgtgggggcagaagcaagcg -3' SEQ ID NO: 17

3'- gatccgcttgcttctgcccccacagtgtatcccttcttcagg-5' SEQ ID NO: 18

5'-gatcccgggcactgaagtatgaagtgcaaggcgaag -3' SEQ ID NO: 19

3'- gatccttcgccttgcacttcatacttcagtgcccgg-5' SEQ ID NO: 20

5'-gatcctagtgggagacattggaaatgtgaacatgtgggactttgtgg -3' SEQ ID NO: 21

3'- gatcccacaaagtcccacatgttcacatttccaatgtctcccactag-5' SEQ ID NO: 22

【0012】

EXAMPLE 3

Expression and Extraction of Fused Enzymes G308C1, G308C2, G308C3, G308C5 and G308C13

Recombinant DNAs pUCMG308C1, pUCMG308C3, pUCMG308C5, pUCMG308C2 and pUCMG308C13 of Example 2 were transformed into E. Coli XL1-Blue. According to the method of Levy et al. (Protein Science, 1, 329- (1992)), the transformed E. Coli was shake cultured in LB medium (DIFCO) at 37°C for 16 hours to obtain a culture medium. The resulting culture medium was inoculated in LB medium to yield a final concentration of 2%, and shake cultured at 37°C for 5 hours. Then, IPTG (isopropyl- β -D-thiogalactopyranoside) was added to achieve a final concentration of 0.5 mM, thereby inducing expression, and the culture was incubated at 37°C for 16 hours, followed by centrifugation at 4°C at 6000 rpm for 10 minutes to

obtain the cells. The cells were suspended in a 10 mM Tris/HCl buffer containing 8% sucrose, 0.1% Triton-X and 50mM EDTA, and lysozyme was added thereto so as to give a final concentration of 33 mg/ml. The resulting product was allowed to stand at 37°C for 30 minutes, and then, insoluble material was removed by centrifugation at 15000 rpm for 20 minutes to obtain a solution of each of fused enzymes G308C1, G308C2, G308C3, G308C5 and G308C13. As a control, a wild type enzyme solution was also similarly obtained from plasmid pUCMG.

【0013】

EXAMPLE 4

Effect of Anti-CRP Antibody to Enzyme Activity of Fused Enzymes G308C1, G308C2, G308C3, G308C5 and G308C13

The activity of the fused enzyme solutions obtained in Example 3 was assayed in the absence and presence of an anti-CRP antibody, respectively, to examine modulations in enzyme activity due to the binding of the anti-CRP antibody. To 6 μl of a 400-fold dilution of each fused enzyme solution with a 100 mM Tris/HCl buffer (pH 7.8) containing 1% bovine albumin, 3 mM magnesium chloride and 150 mM sodium chloride, 150 μl of a 100 mM Tris/HCl buffer containing 3 mM magnesium chloride and 150 mM sodium chloride (pH 7.8, buffer A), or of a 100-fold antibody dilution of anti-CPR goat antibody with buffer A was added, followed by reaction at 37°C for 5 minutes. Then, 75 μl of buffer A containing 10 mM glucose-6-phosphate (G6P) and 6 mM nicotinamide adenine dinucleotide (NAD) was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes were determined as G6PDH activity. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody to that in the absence of the antibody is shown in Table 2. As a result, it is known that the wild type G6PDH indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzymes G308C1, G308C2,

G308C3, G308C5 and G308C13 are decreased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof. That is to say, in the fused enzymes in which CRP peptides represented by SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 or a part thereof are inserted at Pro308/Ala309 of G6PDH, it is found that their enzyme activity is inhibited by the binding of the anti-CRP antibody.

【Table 2】

	Activity Ratio
Wild type	101%
Fused Enzyme G308C1	86%
G308C2	29%
G308C3	36%
G308C5	88%
G308C13	39%

Activity ratio (%) = (G6PDH activity in the presence of antibody)/(G6PDH activity in the absence of antibody) X 100

EXAMPLE 5

Assay of Anti-CRP antibody Using Fused Enzyme G308C1

Modulations in the activity of fused enzyme G308C1 which are dependent on the amount of the anti-CRP antibody were examined. To 50 μl of each of 100-fold, 1000-fold, 10000-fold and 100000-fold dilutions of the anti-CRP monoclonal antibody with buffer A, or to 50 μl of buffer A, 100 μl of a 3300-fold dilution of fused enzyme G308C1 diluted with buffer A was added, followed by reaction at 37°C for 5 minutes. Then, 75 μl of buffer A containing 10 mM G6P and 6 mM NAD was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes were determined as G6PDH activity. Results thereof are shown in Fig. 1. As known from the results, a phenomenon is observed that the enzyme activity increases with a decrease in the amount of the anti-CRP antibody. That is to say, it is shown that the anti-CRP antibody can be assayed using

the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Pro308/Ala309 of G6PDH.

【0014】

EXAMPLE 6

Assay of CRP Using Fused Enzyme G308C1

It was examined whether the hybrid enzyme activity inhibited by the binding of the anti-CRP antibody is recovered with an increase in the CRP concentration. To 6 μl of each of CRP solutions of various concentrations (0, 10, 20 and 40 mg/dl), 100 μl of a 3300-fold dilution of a solution of fused enzyme G308C1 diluted with buffer A was added, followed by reaction at 37°C for 3 minutes. Then, 50 μl of a 10000-fold dilution of the anti-CRP monoclonal antibody diluted with buffer A was added thereto. After further reaction at 37°C for 3 minutes, 75 μl of buffer A containing 10 mM G6P and 6 mM NAD was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes were determined as G6PDH activity. As shown in Fig. 2, a phenomenon is observed that the activity is recovered with an increase in CRP concentration. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Pro308/Ala309 of G6PDH.

EXAMPLE 7

Assay of CRP Using Fused Enzymes G308C2, G308C3 and G308C5

The assay of CRP was tried in the same manner as with Example 6. Dilutions of fused enzymes G308C2, G308C3 and G308C5 were 5000-fold, 15000-fold and 2000-fold, respectively, and the dilution ratio of anti-CPR goat antibody was 8-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in CRP concentration, as shown in Figs. 3,4 and 5. That is to say, it is shown that CRP can be assayed using the fused enzymes in which CRP peptides represented by SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 are each inserted at Pro308/Ala309 of

G6PDH.

【0015】

EXAMPLE 8

Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Inserted at Asp306/Val307 of G6PDH

Using a combinations of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 23 and a combination of the oligonucleotide primers represented by SEQ ID NO: 12 and SEQ ID NO: 24, recombinant pUCMG306B having a BamH I site only at Asp306/Val307 of the G6PDH gene was constructed, and the synthetic oligonucleotides represented by SEQ ID NO: 13 and SEQ ID NO: 14 were ligated thereto to construct recombinant DNA pUCMG306C1, in the same manner as with Example 2.

EXAMPLE 9

Assay of CRP Using Fused Enzyme G306C1

A solution of fused enzyme G306C1 was obtained in the same manner as with Example 3. Then, the assay of CRP was tried in the same manner as with Example 6. A 500-fold dilution of the fused enzyme solution was used, and the dilution ratio of anti-CPR monoclonal antibody was 3200-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in CRP concentration, as shown in Fig. 6. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Asp306/Val307 of G6PDH.

【0016】

EXAMPLE 10

Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Inserted between Ala309/Asp310 of G6PDH

Using a combinations of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 25 and a combination of the oligonucleotide primers

represented by SEQ ID NO: 12 and SEQ ID NO: 26, recombinant pUCMG309B having a BamH I site only at Ala309/Asp310 of the G6PDH gene was constructed, and the synthetic oligonucleotides represented by SEQ ID NO: 13 and SEQ ID NO: 14 were ligated thereto to construct recombinant DNA pUCMG309C1, in the same manner as with Example 2.

EXAMPLE 11

Assay of CRP Using Fused Enzyme G309C1

A solution of fused enzyme G309C1 was obtained in the same manner as with Example 3. Then, the assay of CRP was tried in the same manner as with Example 6. A 2500-fold dilution of the fused enzyme solution and a 1000-fold dillution of anti-CRP monoclonal antibody were used, and the dilution ratio of anti-CPR monoclonal antibody was 10000-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in CRP concentration, as shown in Fig. 7. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Ala309/Asp310 of G6PDH.

【0017】

EXAMPLE 12

Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Added to C-Terminal of G6PDH

Using plasmid pBSMG, the oligonucleotide primer represented by SEQ ID NO: 27 and Mutan-K (Takara Shuzo Co., Ltd.), plasmid pBSMGCB having a restriction enzyme BamH I sequence added to the C-terminal of the G6PDH gene was constructed according to the Kunkel method. Then, recombinant pUCMGCB having the restriction enzyme BamH I sequence only at the C-terminal was constructed, and the synthetic oligonucleotide represented by SEQ ID NO: 13 was ligated thereto to construct recombinant DNA pUCMGCC1, in the same manner as with Examples 1 and 2.

EXAMPLE 13

Effect of Anti-CRP Antibody to Enzyme Activity of Fused Enzyme GCC1

A solution of fused enzyme GCC1 was obtained in the same manner as with Example 3. Then, the effect at the time when the anti-CRP antibody was ligated to fused enzyme GCC1 was examined by the same procedure as with Example 4. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody to that in the absence of the antibody is shown in Table 3. As a result, it is known that the wild type G6PDH indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzyme GCC1 is decreased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof. That is to say, in the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is ligated to the C-terminal of G6PDH, it is found that its enzyme activity is inhibited by the binding of the anti-CRP antibody.

【Table 3】

	Activity Ratio
Wild type	101%
Fused Enzyme GCC1	95%

Activity ratio (%) = (G6PDH activity in the presence of antibody)/(G6PDH activity in the absence of antibody) X 100

【0018】

EXAMPLE 14

Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Ligated between Glu362/Gln363 of G6PDH

Using a combinations of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 28 and a combination of the oligonucleotide primers represented by SEQ ID NO: 12 and SEQ ID NO: 29, recombinant pUCMG362B having a restriction enzyme BamH I sequence only at Glu362/Gln363 of the G6PDH gene was

constructed, and the synthetic oligonucleotides represented by SEQ ID NO: 13 and SEQ ID NO: 14 were ligated thereto to construct recombinant DNA pUCMG362C1, in the same manner as with Example 2.

EXAMPLE 15

Assay of Anti-CRP Antibody Using Fused Enzyme G362C1

A solution of fused enzyme G362C1 was obtained in the same manner as with Example 3. Then, modulations in the activity of fused enzyme G362C1 with the amount of the anti-CRP antibody were examined in the same manner as with Example 5. As a result, a phenomenon is observed that the activity is increased with an increase in the anti-CRP antibody concentration, as shown in Fig. 8. That is to say, it is shown that an anti-CRP antibody can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Glu362/Gln363 of G6PDH.

【0019】

EXAMPLE 16

Fused Enzyme Having a Part of CRP-Derived Peptide Represented by SEQ ID NO: 2 Inserted into G6PDH

Synthetic nucleotides (a combination of SEQ ID NO: 32 and SEQ ID NO: 33, and a combination of SEQ ID NO: 34 and SEQ ID NO: 35) having DNA sequence coding for a part of the amino acid sequence represented by SEQ ID NO: 2 were each ligated to recombinant pUCMG306B having a restriction enzyme BamH I sequence only at Asp306/Val307 of the G6PDH gene constructed in Example 8 to construct recombinant DNAs pUCMG308C15 and pUCMG308C18 respectively. Similarly, using recombinant pUCMG308B in Example 2, recombinant DNAs pUCMG308C15 and pUCMG308C18 were each constructed. Using these, fused enzymes G306C15, G306C18, G308C15 and G308C18 were obtained by the same procedure as with Example 3. As to these fused enzymes and fused enzymes G306C1 and G308C1 obtained in Example 9 and Example 3, the effect at the time when the anti-CRP

antibody was ligated to each fused enzyme was examined by the same procedure as with Example 4. The enzyme activity in the presence of the anti-CRP antibody (a 1000-fold dilution of the anti-CRP monoclonal antibody) to that in the absence of the antibody is shown in Table 4 as the activity ratio. Then, using fused enzymes G306C1, G306C15 and G306C18, a comparison of CRP assaying sensitivity was made. To 10 μl of each of CRP solutions having various concentrations (0, 5, 10, 20 and 40 mg/dl), 250 μl of a mixed solution of each fused enzyme and the anti-CRP monoclonal antibody diluted with buffer A was added, followed by reaction at 37°C for 5 minutes. Then, 125 μl of buffer A containing 10 mM G6P and 6 mM NAD was added thereto, followed by further reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 340 nm for 5 minutes were determined as G6PDH activity. The dilution ratios of fused enzymes G306C1, G306C15 and G306C18 were 1040-fold, 6240-fold and 58500-fold, respectively, and all the dilution ratio of anti-CPR monoclonal antibody was 15600-fold. Results thereof are shown in Fig. 9. The results show the possibility of controlling the assaying sensitivity by the selection of the enzyme site into which the peptide is inserted, and/or by the selection of the peptide length to be inserted.

【Table 4】

	Activity Ratio
Wild type	100.4%
Fused Enzyme G306C1	53.1%
G306C15	54.0%
G306C18	16.2%
G308C1	78.9%
G308C15	89.8%
G308C18	94.6%

CRP peptide sequences inserted into the respective fused enzymes are shown below:

G306C1, G308C1;

Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser Asp Thr Ser

G306C15, G308C15;

Asp Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu Ser

G306C18, G308C18;

Arg Lys Ala Phe Val Phe Pro Lys Glu Ser

【0020】

EXAMPLE 17

Fused Enzymes Having CRP-Derived Peptides Inserted into N-Terminal, at Asp294/Ser295 and at Leu302/Glu303 of G6PDH

Using plasmid pBSMG, the oligonucleotide primer represented by SEQ ID NO: 36 and Mutan-K (Takara Shuzo Co., Ltd.), plasmid pBSMGNB having a restriction enzyme BamH I sequence added to the N-terminal of the G6PDH gene was constructed according to the Kunkel method. Then, recombinant pUCMGNB having the restriction enzyme BamH I sequence only at the N-terminal was constructed by the same procedure as with Examples 1 and 2. Further, using a combination of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 37 and a combination of the oligonucleotide primers represented by SEQ ID NO: 12 and SEQ ID NO: 38, recombinant pUCMG294B having a restriction enzyme BamH I sequence only at Asp294/Ser295 of the G6PDH gene was constructed, and using a combination of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 39 and a combination of the oligonucleotide primers represented by SEQ ID NO: 12 and SEQ ID NO: 40, recombinant pUCMG302B having a restriction enzyme BamH I sequence only at Leu302/Glu303 of the G6PDH gene was constructed, by the same procedure as with Example 2. Then, the synthetic polynucleotides represented by SEQ ID NO: 34 and SEQ ID NO: 35 were ligated thereto to construct recombinant DNAs pUCMGNC18, pUCMG294C18 and pUCMG302C18. Solutions of fused enzymes GNC18, G294C18 and G302C18 were obtained by the same procedure as with Example 3. The effect at the time when the anti-CRP antibody was bound to each fused enzyme was examined

by the same procedure as with Example 4. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody (a 1000-fold dilution of the anti-CRP monoclonal antibody) to that in the absence of the antibody is shown in Table 5. As a result, it is known that the wild type G6PDH indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzymes GNC18, G294C18 and G302C18 are decreased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof. That is to say, in all the fused enzymes in which CRP peptides are inserted into the N-terminal, at Asp294/Ser295 and at Leu302/Glu303 of G6PDH, it is found that their enzyme activity is inhibited by the binding of the anti-CRP antibody.

【Table 5】

	Activity Ratio
Wild type	100.3%
Fused Enzyme GNC18	90.2%
G294C18	51.4%
G302C18	57.5%

【0021】

EXAMPLE 18

Fused Enzyme Having CRP-Derived Peptides Inserted into Two Positions, at Asp306/Val307 and C-Terminal of G6PDH

The synthetic oligonucleotides represented by SEQ ID NO: 32 and SEQ ID NO: 33 were ligated to recombinant pUCMGCB constructed in Example 12 to construct recombinant DNA pUCMGCC15 by the same procedure as with Example 2, and this recombinant DNA was digested with restriction enzymes Bpu1102 I and Pst I to recover an about 0.5-kbp fragment. Further, recombinant DNA pUCMG306C18 constructed in Example 16 was digested with restriction enzymes Nco I and Bpu1102 I to recover an about 1.0-kbp fragment. Still further, recombinant DNA pUCMG constructed in Example 1 was digested with restriction enzymes Nco I and Pst I to recover an about

2.7-kbp fragment. These three recovered fragments were ligated, thereby constructing recombinant DNA pUCMG306C18+CC15 in which synthetic oligonucleotides coding for CRP-derived peptide were inserted into two positions of the G6PDH gene. By the same procedure as with Example 3, a solution of fused enzyme G306C18+CC15 was obtained. The effect at the time when the anti-CRP antibody was bound to the fused enzyme was examined by the same procedure as with Example 4. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody (a 1000-fold dilution of the anti-CRP monoclonal antibody) to that in the absence of the antibody is shown in Table 6. As a result, it is known that the wild type G6PDH indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzyme G306C18+CC15 is decreased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof. That is to say, in the fused enzyme in which a part of CRP peptide represented by SEQ ID NO: 2 are inserted into two positions, between Asp306/Val307 and the C-terminal, it is found that its enzyme activity is inhibited by the binding of the anti-CRP antibody.

【Table 6】

	Activity Ratio
Wild type	99.9%
Fused Enzyme G306C18+CC15	15.2%

【0022】

EXAMPLE 19

Fused Enzyme Having Human CRP-Derived Peptide Substituted for a part from Val307 to Ala309 of G6PDH

The about 0.9-kbp DNA fragment containing from the N-terminal to Asp306 of the G6PDH gene, in which the restriction enzyme BamH I sequence was added to a downstream site, prepared in Example 8, and the about 0.6-kbp DNA fragment containing from Asp310 to the C-terminal of the G6PDH gene, in which the restriction

enzyme BamH I sequence was added to an upstream site, prepared in Example 10, were used with each other to construct recombinant pUCMG306d3B in which the restriction enzyme BamH I sequence was substituted for a part from Val307 to Ala309 of the G6PDH gene, by the same procedure as with Example 2. The synthetic oligonucleotides represented by SEQ ID NO: 13 and SEQ ID NO: 14 were ligated thereto to construct recombinant DNA pUCMG306d3C1. A solution of fused enzyme G306d3C1 was obtained by the same procedure as with Example 3. Then, CRP was assayed in the same manner as with Example 6. A 5000-fold dilution of the fused enzyme solution was used, and the dilution ratio of anti-CPR monoclonal antibody was 10000-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in CRP concentration, as shown in Fig. 10. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is substituted for from Val307 to Ala309 of G6PDH.

【0023】

Example 20

Fused Enzyme Having Tyr Substituted for Asp306 of G6PDH and CRP-Derived Peptide Ligated at Tyr306/Val307 Without Addition of Recognition Sequence for Restriction Enzyme

Using a combinations of the oligonucleotide primers represented by SEQ ID NO: 7 and SEQ ID NO: 41 and a combination of the oligonucleotide primers represented by SEQ ID NO: 12 and SEQ ID NO: 42, recombinant pUCMG306E having a restriction enzyme Eco105 I sequence as a sequence coding for Tyr306/Val307 by substituting Tyr for Asp306 of the G6PDH gene was constructed, and the synthetic oligonucleotides represented by SEQ ID NO: 43 and SEQ ID NO: 44 were ligated thereto to construct recombinant DNA pUCMG306EC18, by the same procedure as with Example 2. By the same procedure as with Example 3, a solution of fused enzyme G306EC18 was obtained. Then, the effect at the time when the anti-CRP

antibody was ligated to the fused enzyme was examined by the same procedure as with Example 4. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody (a 1000-fold dilution of the anti-CRP monoclonal antibody) to that in the absence of the antibody is shown in Table 7. As a result, it is known that the wild type G6PDH indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzyme G306EC18 is decreased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof. That is to say, in the fused enzyme in which Tyr is substituted for Asp306 of G6PDH and a part of CRP peptide represented by SEQ ID NO: 2 is inserted at Tyr306/Val307, it is found that its enzyme activity is inhibited by the binding of the anti-CRP antibody.

【Table 7】

	Activity Ratio
Wild type	101.0%
Fused Enzyme G306EC18	85.0%

【0024】

EXAMPLE 21

Fused Enzyme Having Hepatitis B Virus preS2 Antigen-Derived Peptide Inserted at Asp306/Val307 of G6PDH

The synthetic oligonucleotides represented by SEQ ID NO: 47 and SEQ ID NO: 48, which code for a partial sequence (SEQ ID NO: 46) of preS2 antigen represented by SEQ ID NO: 45 (S. Usuda, et al., J. Virol. Methods, 80, 97-112 (1999)), were ligated to pUCMG306B prepared in Example 8 to construct recombinant DNA pUCMG306H1. A solution of fused enzyme G306H1 was obtained in the same manner as with Example 3. Then, modulations in the activity of fused enzyme G306H1 with the amount of the anti-preS2 antibody were examined in the same manner as with Example 5. As a result, a phenomenon is observed that the enzyme activity

decreases with an increase in the amount of the anti-preS2 antibody, as shown in Fig. 11. That is to say, it is shown that the anti-preS2 antibody can be assayed using the fused enzyme in which the preS2 peptide represented by SEQ ID NO: 46 is inserted at Asp306/Val307 of G6PDH.

【0025】

EXAMPLE 22

Fused Enzyme Having Parathyroid Hormone (PTH)-Derived Peptide Inserted at Asp306/Val307 of G6PDH

The synthetic oligonucleotides represented by SEQ ID NO: 51 and SEQ ID NO: 52, which code for a partial sequence (SEQ ID NO: 50) of PTH represented by SEQ ID NO: 49 (J. H. Habener et al., Metabolic Bone Disease, 2nd Edition, 69, WB Saunders, Philadelphia (1999)), were ligated to pUCMG306B prepared in Example 8 to construct recombinant DNA pUCMG306P1. A solution of fused enzyme G306P1 was obtained in the same manner as with Example 3. Then, PTH was assayed in the same manner as with Example 6. A 2000-fold dilution of the fused enzyme solution was used, and the dilution ratio of anti-PTH monoclonal antibody was 8000-fold. As a result, a phenomenon is observed that the activity is recovered with an increase in the PTH concentration, as shown in Fig. 12. That is to say, it is shown that PTH can be assayed using the fused enzyme in which the PTH peptide represented by SEQ ID NO: 50 is inserted at Asp306/Val307 of G6PDH.

【0026】

EXAMPLE 23

Construction of Plasmid Containing β-Galactosidase Gene

E. coli ATCC 25922 was inoculated into 3 ml of LB media (DIFCO), and shake cultured at 37°C for 16 hours to obtain a culture medium. Then, the culture medium was centrifuged at 4°C at 6000 rpm for 10 minutes, and harvested to obtain cells. Genomic DNA of E. coli was extracted from the resulting cells according to a

conventional method described in Molecular Cloning (J. Sambrook et al., 2nd Edition, Cold Spring Harbor Laboratory). Then, for obtaining the β-galactosidase gene, the PCR was conducted according to the following procedure. That is to say, 10 ng of the above-mentioned genomic DNA as a template DNA and 0.1 nmol of each of the oligonucleotide primers represented by SEQ ID NO: 53 and SEQ ID NO: 54 which contain the N-terminal and C-terminal sequences of the β-galactosidase gene respectively, which is described in A. Kalnins et al., EMBO J., 2, 593- (1983), were added. Then, the reaction cycle at 94°C for 30 seconds, at 60°C for 1 minute and at 72°C for 5 minutes was repeated 25 times using a DNA thermal cycler (Perkin Elmer) to amplify DNA fragment. As a result, an about 3.1-kbp DNA fragment containing the β-galactosidase gene was specifically amplified. On the other hand, cloning vector pUC19 was digested with Eco RI and Hind III, and an end thereof was made flush with a Klenow fragment. The DNA fragment obtained by the PCR was ligated thereto to construct plasmid pUCB which was cloned in the state that a gene coding for β-galactosidase could be expressed.

【0027】

EXAMPLE 24

Construction of Recombinant DNA Coding for Fused Enzyme Having CRP-Derived Peptide Inserted at Val796/Ser797 of β-galactosidase

Using plasmid pUCB obtained in Example 23 as a template, and using the oligonucleotide primers represented by SEQ ID NO: 53 and SEQ ID NO: 55 and the oligonucleotide primers represented by SEQ ID NO: 54 and SEQ ID NO: 56, the PCR was conducted in the same manner as with Example 2 to obtain an about 2.4-kbp DNA fragment and an about 0.7-kbp DNA fragment in which restriction enzyme BamH I sequences were added at a site downstream from Val796 of β-galactosidase gene and at a site upstream from Ser797, respectively. The fragment of the N-terminal side was digested with restriction enzymes Sac I and BamH I to obtain an about 0.4-kbp DNA

fragment, and the fragment of the C-terminal side was digested with restriction enzymes BamH I and Nde I to obtain an about 0.6-kbp DNA fragment. These fragments were ligated to an about 4.8-kbp DNA fragment obtained by digesting plasmid pUCB with restriction enzymes Sac I and Nde I to construct recombinant pUCB796B having a restriction enzyme BamH I sequence only at Val796/Ser797 of the β-galactosidase gene. The synthetic polynucleotides represented by SEQ ID NO: 13 and SEQ ID NO: 14 were ligated thereto to construct pUCB796C1.

EXAMPLE 25

Effect of Anti-CRP Antibody to Enzyme Activity of Fused Enzyme B796C1

A solution of fused enzyme B796C1 was obtained by the same procedure as with Example 3 with the exception that pUCB796C1 was used as the recombinant. Then, the β-galactosidase activity of the fused enzyme solution was assayed in the absence and presence of the anti-CRP antibody, respectively, according to the method of Villaverde et al. (FEBS Letters, 434, 23- (1998)), to examine modulations in enzyme activity due to the binding of the anti-CRP antibody. To 6 μl of a 50-fold dilution of the fused enzyme solution with a 100 mM phosphate buffer containing 0.1 M 2-mercaptoethanol and 1.0 mM magnesium chloride (pH 7.3, hereinafter referred to as buffer B for brevity), 150 μl of buffer B or a 7500-fold dilution of the anti-CRP monoclonal antibody diluted with buffer B was added, followed by reaction at 37°C for 5 minutes. Then, 24 μl of buffer B containing 17 μM o-nitrophenyl-β-D-galactopyranocide (hereinafter referred to as solution ONPG for brevity) was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 405 nm for 5 minutes after adding solution ONPG were determined as β-galactosidase activity. Activity ratio of the enzyme activity in the presence of the anti-CRP antibody to that in the absence thereof was taken. As a result, it is known that the wild type β-galactosidase indicates no difference in activity between in the absence of the anti-CRP antibody and in the presence thereof, whereas fused enzyme

B796C1 is increased in the enzyme activity in the presence of the anti-CRP antibody, compared to that in the absence thereof, as shown in Table 8. That is to say, in the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Val796/Ser797 of β-galactosidase, it is found that its enzyme activity is amplified by the binding of the anti-CRP antibody.

【Table 8】

	Activity Ratio
Wild type	100.2%
Fused Enzyme B796C1	170.5%

【0028】

EXAMPLE 26

Assay of Anti-CRP Antibody Using Fused Enzyme B796C1

Modulations in the activity of fused enzyme B796C1 which are dependent on the amount of the anti-CRP antibody were examined. To 50 μl of each of 1000-fold, 10000-fold and 100000-fold dilutions of the anti-CRP monoclonal antibody with buffer B, or to 50 μl of buffer B, 100 μl of a 1250-fold dilution of fused enzyme B796C1 diluted with buffer B was added, followed by reaction at 37°C for 5 minutes. Then, 24 μl of solution ONPG was added thereto, followed by reaction at 37°C for 5 minutes. Then, the changes in absorbance at a wavelength of 405 nm for 5 minutes after adding solution ONPG were determined as β-galactosidase activity. As a result, a phenomenon is observed that the enzyme activity increases with a increase in the amount of the anti-CRP antibody, as shown in Fig. 13. That is to say, it is shown that the anti-CRP antibody can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Val796/Ser797 of β-galactosidase.

EXAMPLE 27

Assay of CRP Using Fused Enzyme B796C1

Using fused enzyme B796C1 and the anti-CRP antibody, CRP was assayed.

To 6 μl of each of CRP solutions having various concentrations (0, 10, 20 and 40 mg/dl), 100 μl of a 1250-fold dilution of a solution of fused enzyme B796C1 diluted with buffer B was added, followed by reaction at 37°C for 3 minutes. Then, 50 μl of a 7500-fold dilution of the anti-CRP monoclonal antibody diluted with buffer B was added thereto. After further reaction at 37°C for 3 minutes, 24 μl of solution ONPG was added thereto, followed by reaction at 37°C for 5 minutes, and the changes in absorbance at a wavelength of 405 nm for 5 minutes after adding solution ONPG were determined as β-galactosidase activity. As a result, a phenomenon is observed that the enzyme activity increased by the binding of the anti-CRP antibody is recovered with an increase in CRP concentration, as shown in Fig. 14. That is to say, it is shown that CRP can be assayed using the fused enzyme in which CRP peptide represented by SEQ ID NO: 2 is inserted at Val796/Ser797 of β-galactosidase.

【0029】

【Effect of the Invention】

Using the hybrid enzyme of the present invention into which the foreign peptide is inserted, the presence and the amount of the material having the binding ability to the foreign peptide can be directly assayed. Further, when the hybrid enzyme and the material having the binding ability to the foreign peptide are used in combination, the presence and the amount of a macromolecule material containing the foreign peptide can be indirectly detected and assayed. Furthermore, the use of the hybrid enzyme of the present invention makes it possible to assay a trace amount of CRP in a sample by a homogeneous colorimetry, which enables early diagnosis of inflammation in the tissue, and further early diagnosis of diseases. Moreover, various macromolecule materials can be easily assayed in homogeneous systems.

【0030】

【Sequencing List】

SEQUENCE LISTING

SEQUENCE LISTING

<110> WAKO PURE CHEMICAL INDUSTRIES, LTD.

<120> Hybrid Enzymes and Use Thereof

<130> WJ018

<140>

<141>

<160> 56

<170> PatentIn Ver. 2.1

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<211> 206

<212> PRT

<213> Human

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35 40 45

Tyr Ser Ile Phe Ser Tyr Ala Thr Lys Arg Gln Asp Asn Glu Ile Leu
50 55 60

Ile Phe Trp Ser Lys Asp Ile Gly Tyr Ser Phe Thr Val Gly Gly Ser
65 70 75 80

Glu Ile Leu Phe Glu Val Pro Glu Val Thr Val Ala Pro Val His Ile
85 90 95

Cys Thr Ser Trp Glu Ser Ala Ser Gly Ile Val Glu Phe Trp Val Asp
100 105 110

Gly Lys Pro Arg Val Arg Lys Ser Leu Lys Lys Gly Tyr Thr Val Gly
115 120 125

Ala Glu Ala Ser Ile Ile Leu Gly Gln Glu Gln Asp Ser Phe Gly Gly
130 135 140

Asn Phe Glu Gly Ser Gln Ser Leu Val Gly Asp Ile Gly Asn Val Asn
145 150 155 160

Met Trp Asp Phe Val Leu Ser Pro Asp Glu Ile Asn Thr Ile Tyr Leu
165 170 175

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Glu Val Gln Gly Glu Val Phe Thr Lys Pro Gln Leu Trp Pro
195 200 205

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<212> PRT

<213> Human

<400> 2

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<210> 3

<211> 27

<212> PRT

<213> Human

<400> 3

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<211> 12

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<400> 4

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<210> 5

<211> 10

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<400> 5

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1 5 10

<210> 6

<211> 486

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20 25 30

Lys Gly Tyr Leu Gln Lys His Phe Ala Ile Val Gly Thr Ala Arg Gln

35 40 45

Ala Leu Asn Asp Asp Glu Phe Lys Gln Leu Val Arg Asp Ser Ile Lys

50 55 60

Asp Phe Thr Asp Asp Gln Ala Gln Ala Glu Ala Phe Ile Glu His Phe
65 70 75 80

Ser Tyr Arg Ala His Asp Val Thr Asp Ala Ala Ser Tyr Ala Val Leu
85 90 95

Lys Glu Ala Ile Glu Glu Ala Ala Asp Lys Phe Asp Ile Asp Gly Asn
100 105 110

Arg Ile Phe Tyr Met Ser Val Ala Pro Arg Phe Phe Gly Thr Ile Ala
115 120 125

Lys Tyr Leu Lys Ser Glu Gly Leu Leu Ala Asp Thr Gly Tyr Asn Arg
130 135 140

Leu Met Ile Glu Lys Pro Phe Gly Thr Ser Tyr Asp Thr Ala Ala Glu
145 150 155 160

Leu Gln Asn Asp Leu Glu Asn Ala Phe Asp Asp Asn Gln Leu Phe Arg
165 170 175

Ile Asp His Tyr Leu Gly Lys Glu Met Val Gln Asn Ile Ala Ala Leu

180 185 190

Arg Phe Gly Asn Pro Ile Phe Asp Ala Ala Trp Asn Lys Asp Tyr Ile
195 200 205

Lys Asn Val Gln Val Thr Leu Ser Glu Val Leu Gly Val Glu Glu Arg
210 215 220

Ala Gly Tyr Tyr Asp Thr Ala Gly Ala Leu Leu Asp Met Ile Gln Asn
225 230 235 240

His Thr Met Gln Ile Val Gly Trp Leu Ala Met Glu Lys Pro Glu Ser
245 250 255

Phe Thr Asp Lys Asp Ile Arg Ala Ala Lys Asn Ala Ala Phe Asn Ala
260 265 270

Leu Lys Ile Tyr Asp Glu Ala Glu Val Asn Lys Tyr Phe Val Arg Ala
275 280 285

Gln Tyr Gly Ala Gly Asp Ser Ala Asp Phe Lys Pro Tyr Leu Glu Glu
290 295 300

Leu Asp Val Pro Ala Asp Ser Lys Asn Asn Thr Phe Ile Ala Gly Glu

305 310 315 320

Leu Gln Phe Asp Leu Pro Arg Trp Glu Gly Val Pro Phe Tyr Val Arg
325 330 335

Ser Gly Lys Arg Leu Ala Ala Lys Gln Thr Arg Val Asp Ile Val Phe
340 345 350

Lys Ala Gly Thr Phe Asn Phe Gly Ser Glu Gln Glu Ala Gln Glu Ala
355 360 365

Val Leu Ser Ile Ile Ile Asp Pro Lys Gly Ala Ile Glu Leu Lys Leu
370 375 380

Asn Ala Lys Ser Val Glu Asp Ala Phe Asn Thr Arg Thr Ile Asp Leu
385 390 395 400

Gly Trp Thr Val Ser Asp Glu Asp Lys Lys Asn Thr Pro Glu Pro Tyr
405 410 415

Glu Arg Met Ile His Asp Thr Met Asn Gly Asp Gly Ser Asn Phe Ala
420 425 430

Asp Trp Asn Gly Val Ser Ile Ala Trp Lys Phe Val Asp Ala Ile Ser

435 440 445

Ala Val Tyr Thr Ala Asp Lys Ala Pro Leu Glu Thr Tyr Lys Ser Gly
450 455 460

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Ala Trp Val Phe Lys Gly
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<223> Description of Artificial Sequence: Oligonucleotide primer

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<223> Description of Artificial Sequence: Oligonucleotide primer

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 2, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

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<210> 14

<211> 54

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Complemental DNA of Sequence 13

<400> 14

gatccggaag tatccgactc tttgggaaac acaaaagcct tcctcgacat gtcg 54

<210> 15

<211> 48

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for partial amino acids of Sequence 3, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

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gatccgtgct gtcaccagat gagattaaca ccatctatct tggcgggg 48

<210> 16

<211> 48

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Complemental DNA of Sequence 15

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<210> 17

<211> 42

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 4, and a partial restriction site of BamHI

consisting of 5'end of "gatcc" and 3'end of "g".

<400> 17

gatccctgaa gaagggatac actgtggggg cagaagcaag cg 42

<210> 18

<211> 42

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Complemental DNA of Sequence 17

<400> 18

gatccgcttg cttctgcccc cacagtgtat cccttcttca gg 42

<210> 19

<211> 36

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Oligonucleotide consisting of the DNA coding for amino acids of Sequence 5, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

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<210> 20

<211> 36

<212> DNA

<213> Artificial Sequence

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<212> DNA

<213> Artificial Sequence

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<211> 47

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Complemental DNA of Sequence 21

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<210> 23

<211> 33

<212> DNA

<213> Artificial Sequence

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<210> 24

<211> 34

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Oligonucleotide Primer

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<210> 25

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<400> 25

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<210> 26

<211> 33

<212> DNA

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<210> 27

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<212> DNA

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<223> Description of Artificial Sequence: Oligonucleotide Primer

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<211> 32

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Oligonucleotide Primer

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<210> 29

<211> 32

<212> DNA

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<210> 30

<211> 1024

<212> PRT

<213> Escherichia coli

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20 25 30

Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro
35 40 45

Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg Phe Ala Trp Phe

50 55 60

Pro Ala Pro Glu Ala Val Pro Glu Ser Trp Leu Glu Cys Asp Leu Pro

65 70 75 80

Glu Ala Asp Thr Val Val Val Pro Ser Asn Trp Gln Met His Gly Tyr

85 90 95

Asp Ala Pro Ile Tyr Thr Asn Val Thr Tyr Pro Ile Thr Val Asn Pro

100 105 110

Pro Phe Val Pro Thr Glu Asn Pro Thr Gly Cys Tyr Ser Leu Thr Phe

115 120 125

Asn Val Asp Glu Ser Trp Leu Gln Glu Gly Gln Thr Arg Ile Ile Phe

130 135 140

Asp Gly Val Asn Ser Ala Phe His Leu Trp Cys Asn Gly Arg Trp Val

145 150 155 160

Gly Tyr Gly Gln Asp Ser Arg Leu Pro Ser Glu Phe Asp Leu Ser Ala

165 170 175

Phe Leu Arg Ala Gly Glu Asn Arg Leu Ala Val Met Val Leu Arg Trp
180 185 190

Ser Asp Gly Ser Tyr Leu Glu Asp Gln Asp Met Trp Arg Met Ser Gly
195 200 205

Ile Phe Arg Asp Val Ser Leu Leu His Lys Pro Thr Thr Gln Ile Ser
210 215 220

Asp Phe His Val Ala Thr Arg Phe Asn Asp Asp Phe Ser Arg Ala Val
225 230 235 240

Leu Glu Ala Glu Val Gln Met Cys Gly Glu Leu Arg Asp Tyr Leu Arg
245 250 255

Val Thr Val Ser Leu Trp Gln Gly Glu Thr Gln Val Ala Ser Gly Thr
260 265 270

Ala Pro Phe Gly Gly Glu Ile Ile Asp Glu Arg Gly Gly Tyr Ala Asp
275 280 285

Arg Val Thr Leu Arg Leu Asn Val Glu Asn Pro Lys Leu Trp Ser Ala
290 295 300

Glu Ile Pro Asn Leu Tyr Arg Ala Val Val Glu Leu His Thr Ala Asp
305 310 315 320

Gly Thr Leu Ile Glu Ala Glu Ala Cys Asp Val Gly Phe Arg Glu Val
325 330 335

Arg Ile Glu Asn Gly Leu Leu Leu Leu Asn Gly Lys Pro Leu Leu Ile
340 345 350

Arg Gly Val Asn Arg His Glu His His Pro Leu His Gly Gln Val Met
355 360 365

Asp Glu Gln Thr Met Val Gln Asp Ile Leu Leu Met Lys Gln Asn Asn
370 375 380

Phe Asn Ala Val Arg Cys Ser His Tyr Pro Asn His Pro Leu Trp Tyr
385 390 395 400

Thr Leu Cys Asp Arg Tyr Gly Leu Tyr Val Val Asp Glu Ala Asn Ile
405 410 415

Glu Thr His Gly Met Val Pro Met Asn Arg Leu Thr Asp Asp Pro Arg
420 425 430

Trp Leu Pro Ala Met Ser Glu Arg Val Thr Arg Met Val Gln Arg Asp
435 440 445

Arg Asn His Pro Ser Val Ile Ile Trp Ser Leu Gly Asn Glu Ser Gly
450 455 460

His Gly Ala Asn His Asp Ala Leu Tyr Arg Trp Ile Lys Ser Val Asp
465 470 475 480

Pro Ser Arg Pro Val Gln Tyr Glu Gly Gly Gly Ala Asp Thr Thr Ala
485 490 495

Thr Asp Ile Ile Cys Pro Met Tyr Ala Arg Val Asp Glu Asp Gln Pro
500 505 510

Phe Pro Ala Val Pro Lys Trp Ser Ile Lys Lys Trp Leu Ser Leu Pro
515 520 525

Gly Glu Thr Arg Pro Leu Ile Leu Cys Glu Tyr Ala His Ala Met Gly
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Asn Ser Leu Gly Gly Phe Ala Lys Tyr Trp Gln Ala Phe Arg Gln Tyr
545 550 555 560

Pro Arg Leu Gln Gly Gly Phe Val Trp Asp Trp Val Asp Gln Ser Leu
565 570 575

Ile Lys Tyr Asp Glu Asn Gly Asn Pro Trp Ser Ala Tyr Gly Gly Asp
580 585 590

Phe Gly Asp Thr Pro Asn Asp Arg Gln Phe Cys Met Asn Gly Leu Val
595 600 605

Phe Ala Asp Arg Thr Pro His Pro Ala Leu Thr Glu Ala Lys His Gln
610 615 620

Gln Gln Phe Phe Gln Phe Arg Leu Ser Gly Gln Thr Ile Glu Val Thr
625 630 635 640

Ser Glu Tyr Leu Phe Arg His Ser Asp Asn Glu Leu Leu His Trp Met
645 650 655

Val Ala Leu Asp Gly Lys Pro Leu Ala Ser Gly Glu Val Pro Leu Asp
660 665 670

Val Ala Pro Gln Gly Lys Gln Leu Ile Glu Leu Pro Glu Leu Pro Gln
675 680 685

Pro Glu Ser Ala Gly Gln Leu Trp Leu Thr Val Arg Val Val Gln Pro
690 695 700

Asn Ala Thr Ala Trp Ser Glu Ala Gly His Ile Ser Ala Trp Gln Gln
705 710 715 720

Trp Arg Leu Ala Glu Asn Leu Ser Val Thr Leu Pro Ala Ala Ser His
725 730 735

Ala Ile Pro His Leu Thr Thr Ser Glu Met Asp Phe Cys Ile Glu Leu
740 745 750

Gly Asn Lys Arg Trp Gln Phe Asn Arg Gln Ser Gly Phe Leu Ser Gln
755 760 765

Met Trp Ile Gly Asp Lys Lys Gln Leu Leu Thr Pro Leu Arg Asp Gln
770 775 780

Phe Thr Arg Ala Pro Leu Asp Asn Asp Ile Gly Val Ser Glu Ala Thr
785 790 795 800

Arg Ile Asp Pro Asn Ala Trp Val Glu Arg Trp Lys Ala Ala Gly His
805 810 815

Tyr Gln Ala Glu Ala Ala Leu Leu Gln Cys Thr Ala Asp Thr Leu Ala
820 825 830

Asp Ala Val Leu Ile Thr Thr Ala His Ala Trp Gln His Gln Gly Lys
835 840 845

Thr Leu Phe Ile Ser Arg Lys Thr Tyr Arg Ile Asp Gly Ser Gly Gln
850 855 860

Met Ala Ile Thr Val Asp Val Glu Val Ala Ser Asp Thr Pro His Pro
865 870 875 880

Ala Arg Ile Gly Leu Asn Cys Gln Leu Ala Gln Val Ala Glu Arg Val
885 890 895

Asn Trp Leu Gly Leu Gly Pro Gln Glu Asn Tyr Pro Asp Arg Leu Thr
900 905 910

Ala Ala Cys Phe Asp Arg Trp Asp Leu Pro Leu Ser Asp Met Tyr Thr
915 920 925

Pro Tyr Val Phe Pro Ser Glu Asn Gly Leu Arg Cys Gly Thr Arg Glu
930 935 940

Leu Asn Tyr Gly Pro His Gln Trp Arg Gly Asp Phe Gln Phe Asn Ile
945 950 955 960

Ser Arg Tyr Ser Gln Gln Gln Leu Met Glu Thr Ser His Arg His Leu
965 970 975

Leu His Ala Glu Glu Gly Thr Trp Leu Asn Ile Asp Gly Phe His Met
980 985 990

Gly Ile Gly Gly Asp Asp Ser Trp Ser Pro Ser Val Ser Ala Glu Phe
995 1000 1005

Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln Leu Val Trp Cys Gln Lys
1010 1015 1020

<210> 31

<211> 448

<212> PRT

<213> Escherichia coli

<400> 31

Thr Pro Glu Met Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile
1 5 10 15

Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala Ala
20 25 30

Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile Leu Leu
35 40 45

Ile Gly Asp Gly Met Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr
50 55 60

Ala Glu Gly Ala Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu
65 70 75 80

Thr Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys Pro
85 90 95

Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala Thr Ala Trp Ser Thr Gly
100 105 110

Val Lys Thr Tyr Asn Gly Ala Leu Gly Val Asp Ile His Glu Lys Asp
115 120 125

His Pro Thr Ile Leu Glu Met Ala Lys Ala Ala Gly Leu Ala Thr Gly
130 135 140

Asn Val Ser Thr Ala Glu Leu Gln Asp Ala Thr Pro Ala Ala Leu Val
145 150 155 160

Ala His Val Thr Ser Arg Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu
165 170 175

Lys Cys Pro Gly Asn Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr
180 185 190

Glu Gln Leu Leu Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly Ala
195 200 205

Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly Glu Trp Gln Gly Lys Thr
210 215 220

Leu Arg Glu Gln Ala Gln Ala Arg Gly Tyr Gln Leu Val Ser Asp Ala
225 230 235 240

Ala Ser Leu Asn Ser Val Thr Glu Ala Asn Gln Gln Lys Pro Leu Leu
245 250 255

Gly Leu Phe Ala Asp Gly Asn Met Pro Val Arg Trp Leu Gly Pro Lys
260 265 270

Ala Thr Tyr His Gly Asn Ile Asp Lys Pro Ala Val Thr Cys Thr Pro
275 280 285

Asn Pro Gln Arg Asn Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp
290 295 300

Lys Ala Ile Glu Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu Gln
305 310 315 320

Val Glu Gly Ala Ser Ile Asp Lys Gln Asp His Ala Ala Asn Pro Cys
325 330 335

Gly Gln Ile Gly Glu Thr Val Asp Leu Asp Glu Ala Val Gln Arg Ala
340 345 350

Leu Glu Phe Ala Lys Lys Glu Gly Asn Thr Leu Val Ile Val Thr Ala
355 360 365

Asp His Ala His Ala Ser Gln Ile Val Ala Pro Asp Thr Lys Ala Pro
370 375 380

Gly Leu Thr Gln Ala Leu Asn Thr Lys Asp Gly Ala Val Met Val Met

385 390 395 400

Ser Tyr Gly Asn Ser Glu Glu Asp Ser Gln Glu His Thr Gly Gln Leu

405 410 415

Arg Ile Ala Ala Tyr Gly Pro His Ala Ala Asn Val Val Gly Leu Thr

420 425 430

Asp Gln Thr Asp Leu Phe Tyr Thr Met Lys Ala Ala Leu Gly Leu Lys

435 440 445

<210> 32

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for partial amino acids of Sequence 2, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 32

gatccgacat gtcgaggaag gcttttgtgt ttcccaaaga gtcgg 45

<210> 33

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 32

<400> 33

gatcccgact ctttgggaaa cacaaaagcc ttcctcgaca tgtcg 45

<210> 34

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for partial amino acids of Sequence 2, and a partial restriction site of

BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 34

gatccaggaa ggcttttgtg tttcccaaag agtcgg 36

<210> 35

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 34

<400> 35

gatcccgact ctttgggaaa cacaaaagcc ttcctg 36

<210> 36

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 36

cacaggaaac agaccatggg atccgtttca gaaatc 36

<210> 37

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 37

ttggatccat caccggcacc atattgtgca cg 32

<210> 38

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 38

aaggatcctc agctgacttc aagccatacc ttg 33

<210> 39

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 39

aaggatccaa ggtatggctt gaagtcagct g 31

<210> 40

<211> 31

<212> DNA

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<223> Description of Artificial Sequence: Oligonucleotide primer

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aaggatccaa ggtatggctt gaagtcagct g 31

<210> 41

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<212> DNA

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<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 41

ggtacgtata attcatcaag gtatggcttg 30

<210> 42

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 42

tatacgtacc tgctgattct aaaaac 26

<210> 43

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for partial amino acids of Sequence 2 .

<400> 43

aggaaggctt ttgtgtttcc caaagagtcg 30

<210> 44

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 43

<400> 44

cgactctttg ggaaacacaa aagccttcct 30

<210> 45

<211> 55

<212> PRT

<213> Hepatitis B virus

<400> 45

Met Gln Trp Asn Ser Thr Ala Phe His Gln Ala Leu Gln Asp Pro Arg
1 5 10 15

Val Arg Gly Leu Tyr Phe Pro Ala Gly Gly Ser Ser Ser Gly Thr Val
20 25 30

Asn Pro Ala Pro Asn Ile Ala Ser His Ile Ser Ser Ile Ser Ala Arg
35 40 45

Thr Gly Asp Pro Val Thr Asn

50 55

<210> 46

<211> 12

<212> PRT

<213> Hepatitis B virus

<400> 46

Asp Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly

1 5 10

<210> 47

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 46, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 47

gatccgaccc gcgtgttcgt ggtctgtatt tcccggctgg tg 42

<210> 48

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 47

<400> 48

gatccaccag ccgggaaata cagaccacga acacgcgggt cc 42

<210> 49

<211> 84

<212> PRT

<213> Human

<400> 49

Ala Val Ser Glu Ile Gln Phe Met His Asn Leu Gly Lys His Leu Ser
1 5 10 15

Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His
20 25 30

Asn Phe Val Ala Leu Gly Ala Ser Ile Ala Tyr Arg Asp Gly Ser Ser
35 40 45

Gln Arg Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His Gln
50 55 60

Lys Ser Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Ile Lys
65 70 75 80

Ala Lys Pro Gln

<210> 50

<211> 15

<212> PRT

<213> Human

<400> 50

Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn
1 5 10 15

<210> 51

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotides consisting of the DNA coding for amino acids of Sequence 50, and a partial restriction site of BamHI consisting of 5'end of "gatcc" and 3'end of "g".

<400> 51

gatccgaacg tgttgaatgg ctgcgtaaaa aactgcagga cgttcataac g 51

<210> 52

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Complemental DNA of Sequence 51

<400> 52

gatccgttat gaacgtcctg cagtttttta cgcagccatt caacacgttc g 51

<210> 53

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 53

tatgaccatg attacggatt cactggcc 28

<210> 54

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 54

ctgcccggtt attattattt ttgacaccag 26

<210> 55

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 55

taggatccta cgccaatgtc gttatccagc g 31

<210> 56

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oligonucleotide primer

<400> 56

ttggatccag tgaagcgacc cgcattgacc 30

【0031】

【Brief Description of the Drawings】

【Fig. 1】 Fig.1 is a graph showing the ability for measuring an anti-CRP antibody of hybrid enzyme G308C1 of the present invention.

【Fig. 2】 Fig.2 is a graph showing the ability for measuring CRP of hybrid enzyme G308C1 of the present invention. In Figs. 2 to 14,

Recovery (%) = {(G6PDH activity at each CRP concentration) – (G6PDH activity at 0 mg/dl of CRP)}/{(G6PDH activity in the absence of antibody) -(G6PDH activity at 0 mg/dl of CRP)} X 100; and

Activity ratio (%)= {G6PDH activity in the presence of antibody/ G6PDH activity in the absence of antibody} X 100.

【Fig. 3】 Fig.3 is a graph showing the ability for measuring CRP of hybrid enzyme G308C2 of the present invention.

【Fig. 4】 Fig.4 is a graph showing the ability for measuring CRP of hybrid enzyme G308C3 of the present invention.

【Fig. 5】 Fig.5 is a graph showing the ability for measuring CRP of hybrid enzyme G308C5 of the present invention.

【Fig. 6】 Fig.6 is a graph showing the ability for measuring CRP of hybrid enzyme G306C1 of the present invention.

【Fig. 7】 Fig.7 is a graph showing the ability for measuring CRP of hybrid enzyme G309C1 of the present invention.

【Fig. 8】 Fig.8 is a graph showing the ability for measuring the anti-CRP of hybrid enzyme G362C1 of the present invention.

【Fig. 9】 Fig.9 is a graph comparing the sensitivities of measuring CRP of hybrid enzymes G306C1, G306C15 and G306C18 of the present invention.

【Fig. 10】 Fig.10 is a graph showing the ability for measuring CRP of hybrid enzyme G306d3C1 of the present invention.

【Fig. 11】 Fig.11 is a graph showing the ability for measuring the anti-preS2 antibody of hybrid enzyme G306H1 of the present invention.

【Fig. 12】 Fig.12 is a graph showing the ability for measuring the PTH of hybrid enzyme G306P1 of the present invention.

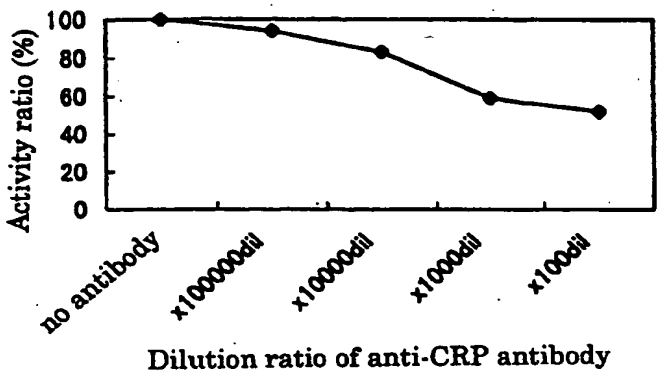
【Fig. 13】 Fig.13 is a graph showing the ability for measuring the anti-CRP of hybrid enzyme B796C1 of the present invention.

【Fig. 14】 Fig.14 is a graph showing the ability for measuring CRP of hybrid enzyme B796C1 of the present invention.

【Title of Document】 Drawing

【Fig.1】

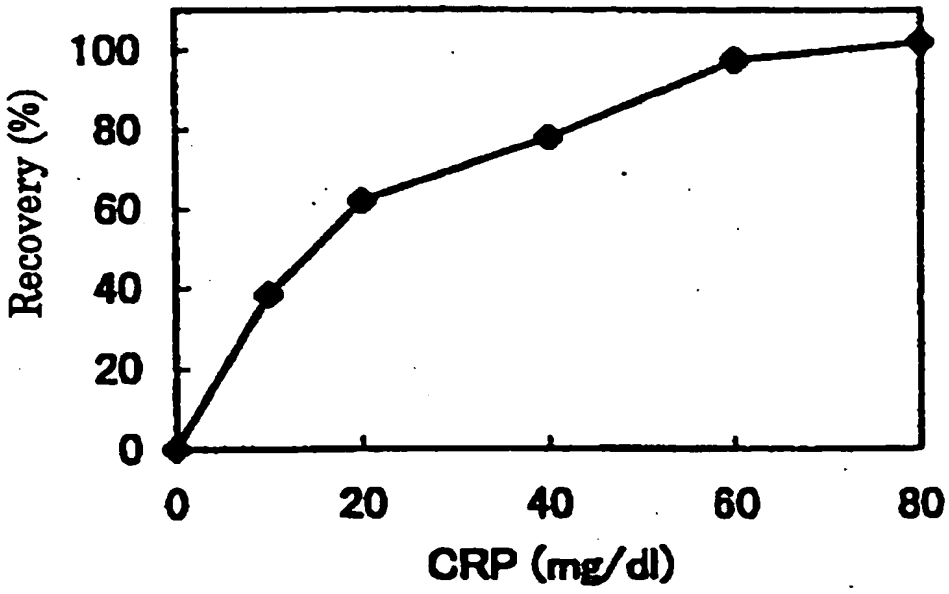
FIG.1



$$\text{Activity ratio (\%)} = \frac{\text{G6PDH activity in the presence of antibody}}{\text{G6PDH activity in the absence of antibody}} \times 100$$

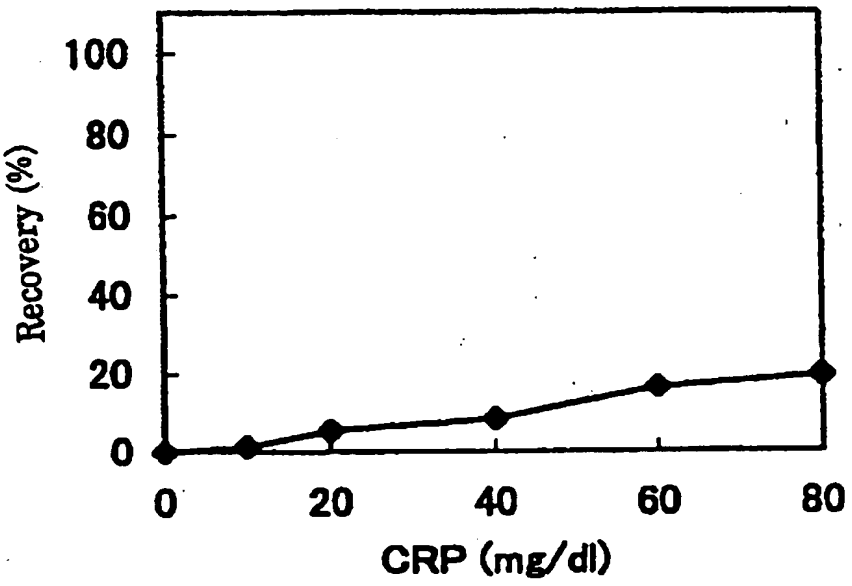
【Fig.2】

FIG.2



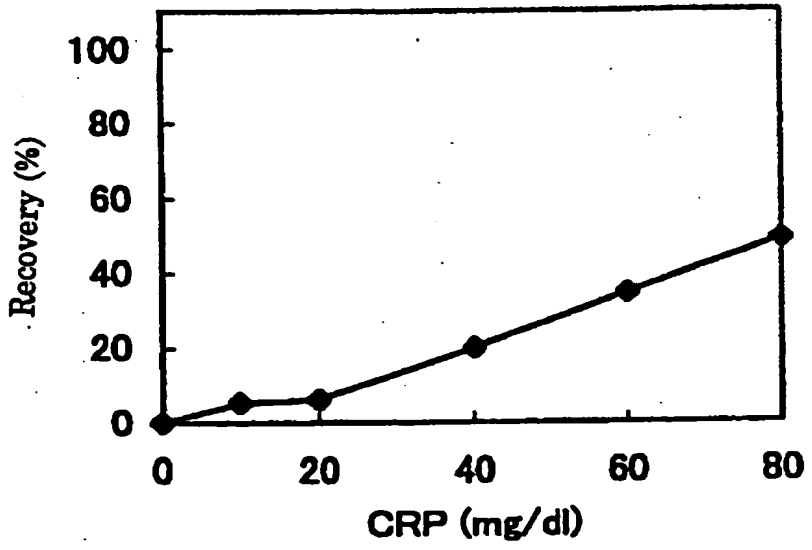
【Fig.3】

FIG.3



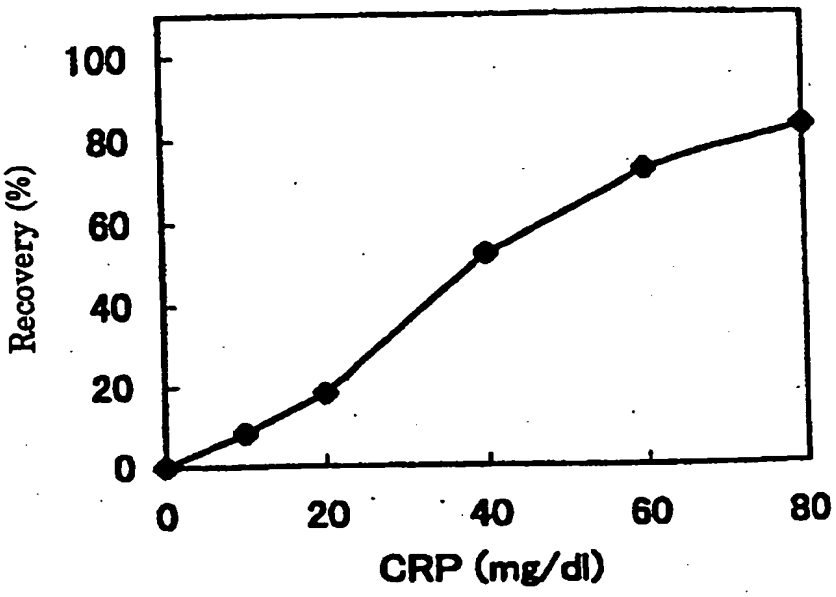
【Fig.4】

FIG.4



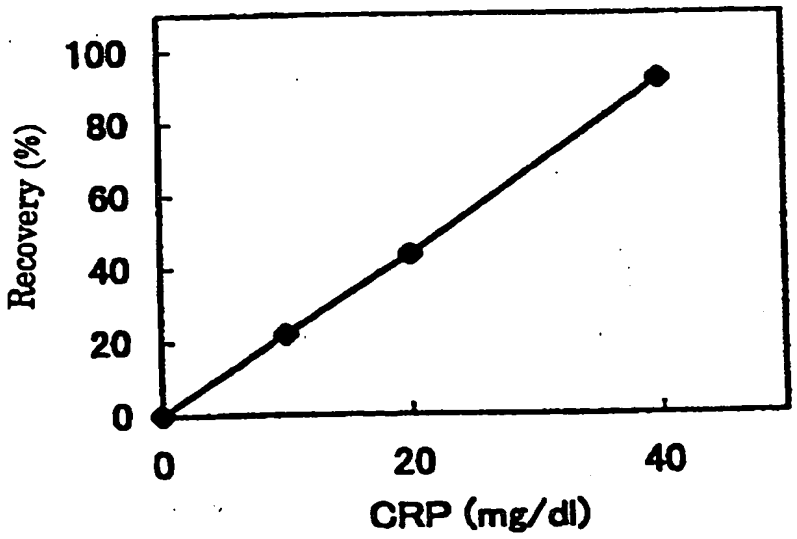
【Fig.5】

FIG.5



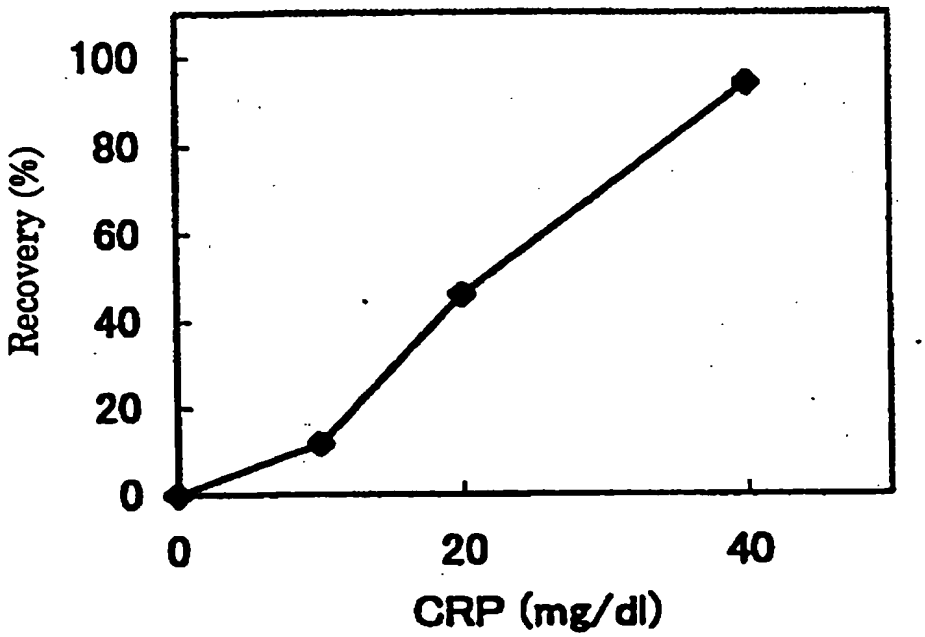
【Fig.6】

FIG.6



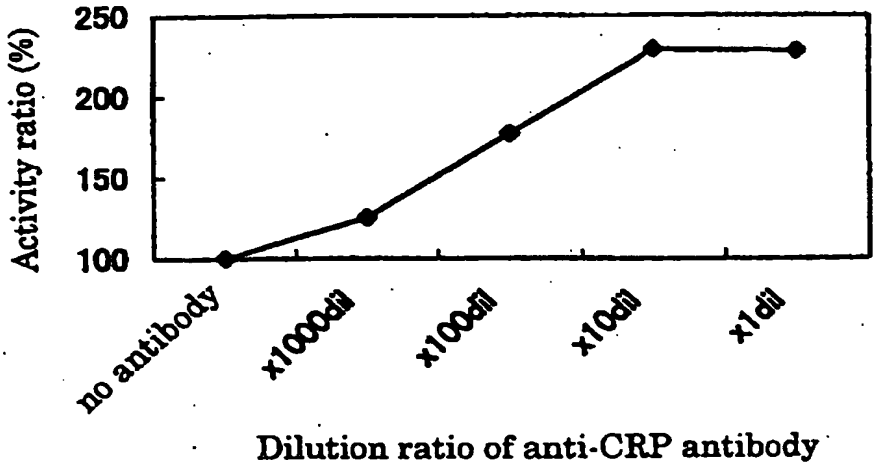
【Fig.7】

FIG.7



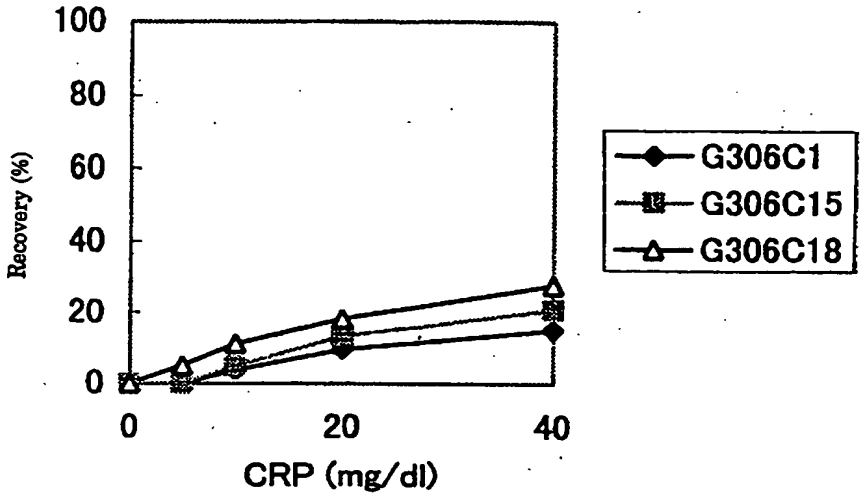
【Fig.8】

FIG.8



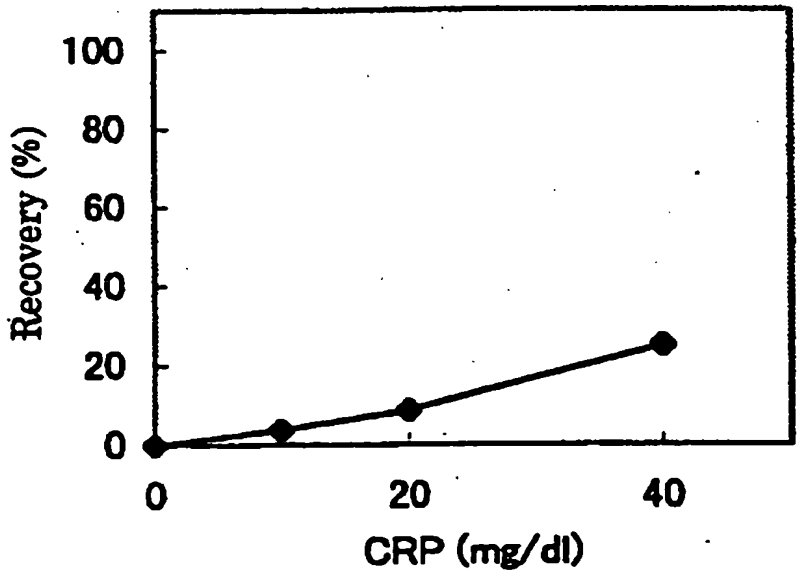
【Fig.9】

FIG.9



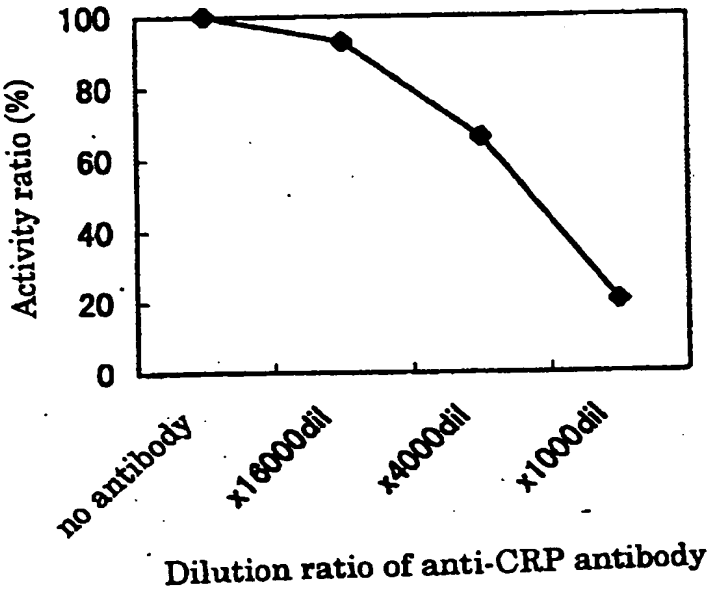
【Fig.10】

FIG.10



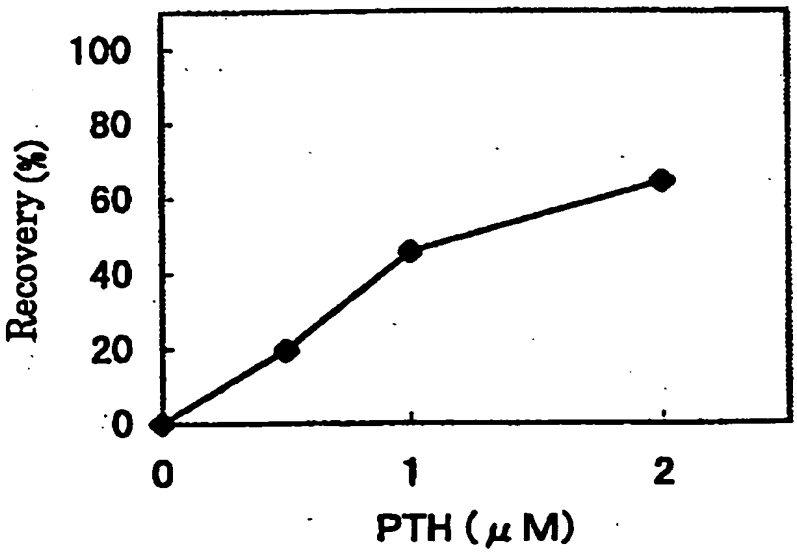
【Fig.11】

FIG.11



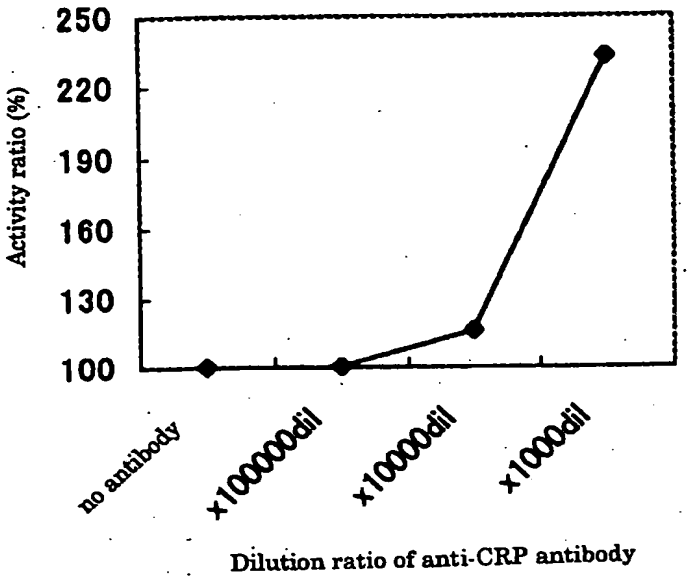
【Fig.12】

FIG.12



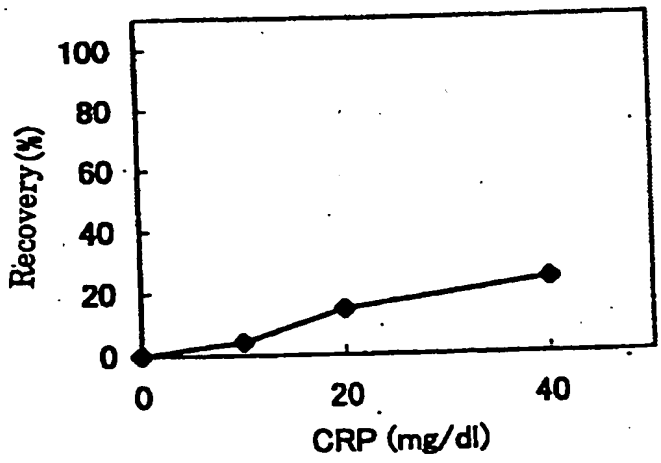
【Fig.13】

FIG.13



【Fig.14】

FIG.14



【Title of Document】 Abstract

【Abstract】

【Subject】 To provide is a method which makes it possible to measure a trace amount of CRP in a sample by a homogeneous colorimetry and a method for assaying a macromolecule material in a homogeneous system.

【Means for Solution】 The present inventors have found the specific positions that the enzyme activity is maintained also when the foreign peptide is inserted in that position, and is modified when a material having binding ability to the inserted foreign peptide is bound to the peptide. The present invention provide the hybrid enzyme which is inserted a CRP-derived peptide into a specific position. By determining the modulation of enzyme activity after contacting the hybrid enzyme with samples, it becomes possible to assay a trace amount of CRP in a sample by a homogeneous colorimetry.

【Selected Drawing】 None

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